JUN 25 1923

VOL. IV. No. 3] [JUNE, 1923.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C.1.

EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, January to December, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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THE EDITORS,
British Journal of Experimental Pathology,
28, GOWER PLACE,
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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE EXCRETION OF "ACETONE" AND NITROGEN IN NAUSEA AND VOMITING OF PREGNANCY.

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Received for publication February 20th, 1923.

THERE have been many theories of the ætiology of the nausea and vomiting of pregnancy, the great majority of which have been based upon clinical observation, and which observations, on the whole, have remained unconfirmed. Of the possible causes to which has been ascribed the origin of this condition, there only remains a certain amount of evidence in favour of a neurosis, or intestinal intoxication, or some metabolic perversion. Observations based upon scientific measurements have been very few, and such as have been made have gone decidedly to discredit most of the theories of the ætiology based upon ordinary clinical findings. In our opinion, however, three contributions stand out prominently, though the general results have led to a negative rather than a positive position.

The first of these was in 1910 by Underhill and Rand (1910). By means of a vigorous criticism of the older work they showed very clearly that it was possible to ascribe all the changes which are to be found in the nitrogen partition of the urine in pernicious vomiting to starvation, and that such alterations as had been observed became, or tended to become, normal by the administration of carbohydrate. They thus ascribed the changes found in the urinary picture to mere lack of carbohydrate due to the starvation of necessity accompanying the vomiting. Though their results were criticised, nevertheless their contention would seem to be established. Losee and Van Slyke (1917) in a general paper on toxæmias of pregnancy published a series of facts upon pernicious vomiting, which showed that this condition was not accompanied by any excess of amino-acids, either in the blood or in the urine, nor that, if acidosis were measured in terms of the bicarbonate reserve of the

body, was any true acidosis to be observed. They did, however, confirm the finding of high ammonia coefficients and low values for urea in the urine. Though they considered these findings to have some diagnostic value, they did not attempt to put any interpretation upon them. Later, Gilliatt and Kennaway (1918), from a study of two cases of pernicious vomiting, were of the opinion that although starvation might perhaps account for some of the changes observed in the urine, this alone was not sufficient. They pointed out that the ammonia coefficient, or, as they expressed it, the ammonia index, might rise very rapidly in pernicious vomiting, whereas such sudden increases were not usual in starvation, and that this coincided with the production of large amounts of acetone bodies; also that after abortion the ammonia index fell with almost equal rapidity. They also drew attention to the scarcity of data on the part played by the acetone bodies in pernicious vomiting.

The general conclusion which could be drawn from these contributions would be that the metabolic disturbance accompanying this condition is of the same essential nature as the disturbance caused by starvation, but is much more intense, although it never reaches such an intensity as to produce any marked disturbance of the acid-base equilibrium.

At about the same time as the publication of the paper of Gilliatt and Kennaway, a theory of the origin of the nausea and vomiting of pregnancy, based upon an essential carbohydrate disturbance, was put forward, which theory was afterwards amplified, and supported by a large mass of clinical observation (Duncan and Harding, 1918; Harding, 1921; Harding and Watson, 1922). The present paper deals with a series of exact measurements upon the urines of a number of cases of pernicious vomiting, taken from the wards of the Toronto General Hospital. The cases range from moderate to severe in type. All were under the direct clinical observation of one of us, and all were treated in essentially the same manner. This treatment in general consisted of rest with isolation, of the administration of carbohydrate to the amount of 50–100 gm. per day, usually by rectum, and the giving of fluids either by mouth (if it were possible for the patient to take them by that route), or interstitially or intravenously. The more detailed management of such cases is to be found in a previous paper. These observations, then, have the advantage of being made on patients who were under a unified control, both from a clinical and a scientific standpoint. We determined the daily excretion of total acetone bodies, total nitrogen, ammonia and urea.* We also determined the total acetone bodies in the blood, and looked for any evidence of "acidosis," by a determination of the CO_2-content of the alveolar air.

Case I (*R*—).

Primipara, aged 23; mild case of occasional vomiting for about two months. Previous history unimportant, though described as of a neurotic type by home physician. Physical examination normal. Patient was able to

* Analytical methods: Acetone, aceto-acetic acid and β-oxybutyric acid by combined method of Van Slyke, and results expressed as total acetone (Van Slyke, 1917; Van Slyke and Fitz, 1917); total nitrogen by method of Kjeldahl-Gunning; ammonia and urea by urease method of Van Slyke and Cullen (1914).

take small amounts of food and to drink water on entering hospital, consequently all that was advised was rest in bed with isolation and light carbohydrate meals. Recovery uneventful.

TABLE I.—*Case I (R—) : Urine Analysis.*

Total acetone in blood ·093 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Dec. 6	525	3·4	·5	2·5	1·3	Light carbohydrate meals.
„ 7	400	4·1	·8	2·4	1·2	„ „
„ 8	820	4·3	·6	3·0	·3	„ „
„ 9	860	3·4	·4	2·5	·1	Protein in diet.
„ 10	350	1·3	·1	1·0	—	„
„ 11	730	3·1	·3	1·3	—	„
„ 12	650	1·9	·1	1·5	—	„

Case II (N—).

Primipara, aged 28; two months pregnant. Nauseated a month; a little vomiting during the previous week. Vomiting then became severe. No food two days previous to admission to hospital. History of biliousness at menstrual periods. Well nourished; negative physical findings, except epigastric pain on pressure. Treatment: Isolation; nutrient enemata, 10 per cent. glucose, 6 oz. *t.i.d.*; fluids urged by mouth. Rapid recovery, and after-history uneventful.

TABLE II.—*Case II (N—) : Urine Analysis.*

Total acetone in blood ·239 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Nov. 24	500	5·0	·5	3·7	4·7	—
„ 25	660	8·0	1·1	5·9	3·5	—
„ 26	755	8·1	1·1	5·7	1·2	Carbohydrate meals.
„ 27	525	5·1	·7	3·2	0·1	„
„ 28	750	5·2	·6	2·8	—	Protein in diet.

Case III (Br—).

Multipara, aged 24. First pregnancy terminated by operation at 7 or 8 months. Second spontaneously aborted at 3 months. Present pregnancy about 3 months. Vomiting started about four weeks before admission to hospital as morning sickness, and had increased. Well nourished, obese woman, rather excitable. Physical examination negative. Treatment: Nutrient enemata, 10 per cent. glucose in saline of 6 oz. *t.i.d.*; bromide gr. xx, chloral gr. x were added to evening enema. Weak tea, lemonade and water urged by mouth. Enemata not well retained by this patient, but when given by Murphy drip the glucose solution was well borne. Recovery in three or four days.

TABLE III.—*Case III (Br—) : Urine Analysis.*

Total acetone in blood ·145 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_2 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Jan. 22	1250	10·4	1·3	7·5	3·7	—
„ 23	1130	6·1	1·0	4·0	0·1	—
„ 24	860	3·8	0·5	2·5	—	Carbohydrate meals.
„ 25	965	5·7	0·8	3·7	—	—

*Case IV (NN—).**

Primipara, aged 19; moderately severe case. Previous history uneventful. Physical findings negative, except for slight icterus. Treatment: Nutrient enemata, 10 per cent. glucose in saline, 6 oz. *t.i.d.* and fluids urged by mouth. Previous to admission patient had been unable to keep down even water, but was easily able to take small amounts of fluid in hospital, and soon augmented her water intake. Clinical condition rapidly improved and patient was eating on the fourth day. After clinical history uneventful. This case is exceptional in that the acetone bodies continued in the urine for a long time, even when the patient was taking large amounts of food, although there was no accompanying clinical manifestation.

TABLE IV.—*Case IV (NN—) : Urine Analysis.*

Total acetone in blood ·172 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Feb. 9	500	7·3	1·7	4·4	6·6	—
„ 10	515	7·0	1·9	3·8	6·9	—
„ 11	525	4·7	1·3	2·5	4·5	—
„ 12	840	4·3	1·3	2·0	4·3	Carbohydrate meals.
„ 13	1250	5·1	1·6	2·3	2·5	„
„ 14	550	2·9	·8	1·1	1·1	„
„ 15	600	2·3	·6	1·0	1·4	„
„ 16	860	2·2	·5	1·0	1·3	„
„ 17	810	3·2	·7	1·7	1·0	Protein in diet.
„ 18	580	1·8	·4	·8	·4	„
„ 19	970	3·0	·5	1·3	·5	„
„ 20	980	2·5	·4	1·3	·5	„
„ 21	500	1·9	·2	1·2	·2	„
„ 22	970	3·4	·5	2·0	·7	„
„ 23	620	2·0	·3	1·2	·4	„
„ 24	820	4·1	·4	2·4	·8	„
„ 25	1210	4·3	·2	2·9	·5	„
„ 26	400	1·5	·2	·9	·1	„
„ 27	620	2·3	·2	1·3	·2	„

* A full report of this case is to be found in a previous paper (Harding and Watson, 1922).

Case V (C—k).

Primipara, aged 32; about three months pregnant. Had been vomiting most of the pregnancy. Of a distinctly nervous type, and gave a history of biliousness at the menstrual periods. No food for the two days previous to entry into hospital. Physical examination normal. Signs of dehydration, however, were present. Treatment: Nutrient enemata of 10 per cent. glucose in saline, with sodium bromide gr. xl, 6 oz. *t.i.d.* This case for the first two or three days did not progress favourably, but the continuance of the enemata and the fluids by mouth produced a sudden change, both clinically and metabolically. This is the only case which showed any evidence of true acidosis, the CO_2 content of the alveolar air being found to be 3·1 per cent. Casts were also found in this urine on two days. After the sudden change on November 9th the patient's condition rapidly improved, although she was subject to occasional mild attacks of nausea.

TABLE V.—*Case V (C—k) : Urine Analysis.*

Total acetone in blood ·115 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Nov. 6	555	6·9	·9	5·0	6·4	—
,, 7	1105	8·0	1·5	5·7	8·0	—
,, 8	1510	6·8	1·6	4·4	8·2	—
,, 9	755	4·2	·7	2·4	·8	Carbohydrate meals. Enemata discontinued.
,, 10	1125	3·2	·4	1·9	—	Protein in diet.
,, 11	1750	4·8	·4	3·4	—	,,

Case VI (M—).

Multipara, aged 35, about 2½ months pregnant. Vomiting for two months previously. Previous two pregnancies had been marked by nausea, but not by excessive vomiting such as this. Patient was a large, florid, well-nourished woman. Slight cough, but chest examination gave negative findings. Tongue and skin moist, lips were cracked, and the patient felt thirsty. Conjunctivæ

TABLE VI.—*Case VI (M—) : Urine Analysis.*

Total acetone in blood ·201 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Feb. 21	370	5·3	1·0	3·4	2·0	—
,, 22	450	4·5	·7	3·2	·7	Interstitial glucose.
,, 23	1080	7·6	·8	5·8	·2	Carbohydrate meals.
,, 24	1260	4·2	·5	2·9	·1	,,
,, 25	2260	5·9	·5	4·1	—	,,
,, 26	2130	3·9	·4	2·6	—	Protein in diet.

were bile-stained; epigastric pain on pressure. Treatment: Nutrient enemata of 10 per cent. glucose in saline, bromide gr. xx, 6 oz. *t.i.d.* This produced a little improvement, but not marked, and on the third day 500 c.c. 10 per cent. sterile glucose in saline were given interstitially. The patient vomited once during the night, and once the day following, but afterwards was free from symptoms. Six days after admission she was able to take low-protein, high-carbohydrate diet. After-history uneventful.

*Case VII (B—k).**

Multipara, aged 27. Previous pregnancy terminated by Cæsarian section at eight months after continuous vomiting. Living child had been obtained. Patient was extremely thin and ill nourished. No jaundice or epigastric pain; vomiting frequent and no food taken for previous week. Physical examination showed a pelvis so contracted that the only possibility of obtaining a living child lay through another Cæsarian section, otherwise negative. Treatment: 10 per cent. glucose in saline 6 oz. *t.i.d.*, with bromide gr. xx and chloral gr. x in evening enema. Fluids urged by mouth. Patient did not improve under this treatment, and on the third day 300 c.c. 10 per cent. glucose in saline were given interstitially. Temporary improvement seemed to follow this, but there was again retrogression, and two days later 1000 c.c. 5 per cent. sterile glucose in saline were given intravenously. This, again, seemed to effect a little improvement, but the change was not lasting. Therapeutic abortion was carried out on February 1st, and the day following the nutrient enemata were recommenced. In two days the patient was well, able to eat small meals, and the acetone bodies had disappeared from the urine.

TABLE VII.—*Case VII (B—k) : Urine Analysis.*

Total acetone in blood ·417 gm. per litre.

Date.	Vol. c.c.	Total N. gm.	NH_3 N. gm.	Urea N. gm.	Total acetone gm.	Remarks.
Jan. 25	400	5·8	·6	4·5	4·7	—
„ 26	570	10·5	1·8	6·9	6·7	—
„ 27	450	7·4	1·4	5·1	5·0	—
„ 28	375	5·9	1·2	3·8	4·3	300 c.c. 10 per cent. glucose interstitially.
„ 29	565	8·3	2·0	4·9	8·0	—
„ 30	260	4·4	·8	2·8	2·0	1000 c.c. 5 per cent. glucose intravenous.
„ 31	525	6·2	·8	4·6	1·5	—
Feb. 1			Abortion.			—
„ 2	1000	7·5	1·2	5·0	2·5	—
„ 3	670	2·3	·2	1·8	—	Carbohydrate meals.

* A full report of this case is to be found in a previous paper (Harding and Watson, 1922).

EXCRETION OF ACETONE BODIES.

The theory which has been put forward in previous papers postulated a carbohydrate disturbance occurring in the maternal liver as the primary factor concerned in the ætiology of the nausea and vomiting of pregnancy with consequent production of acetone bodies in the blood and urine. It has been postulated by Shaffer (1921[1], 1921[2], 1922) and others that the production of acetone bodies is dependent upon a balance between the ketogenic and antiketogenic substances undergoing catabolism. These substances include carbohydrate, protein and fat molecules, but by far the preponderance of antiketogenic molecules is derived from carbohydrate. Whether or no the "acetone bodies" give rise to a disturbance of acid-base equilibrium is a matter which will be discussed later in this paper, and until such a disturbance is shown, it would be better to use the term *ketosis* in this connection. For the present, we wish to discuss their relationship to the clinical findings in the series of patients whose histories we have just, in brief, described. Of the seven cases, six improved rapidly under the treatment, and by the fifth or sixth day were free from all vomiting, and were eating three small meals a day with relish. In all except Case 4 the acetonuria had ceased in a like length of time. These results are typical of all the cases we have seen in which the use of glucose and water, together with rest and isolation, have been successful in overcoming the nausea and vomiting of pregnancy. In Case 4 the acetonuria persisted in a very mild degree for several days after clinical recovery had become an established fact, though previous to this there had been a rapid decline in the total acetone excretion coincident with a rapid improvement in the clinical condition. We have only met with one other case similar to Case 4, in which a mild acetonuria persisted long after the patient was free from symptoms. Case 7 represents the only case upon which we have quantitative data where the treatment failed to produce an improvement in the clinical condition. In spite of the fact that glucose was given both by the interstitial and intravenous route, as well as by rectum, the patient was vomiting as freely at the end of seven days as at the beginning. Here the acetone bodies fluctuate markedly in contrast to the other cases. On the sixth and seventh day it would seem as though they were going to disappear from the urine; the third and fourth days, however, had been marked with a progressive fall in their excretion, only to be followed on the fifth day by a rise to a greater height. Thus, an extremely vigorous administration of glucose in this case failed to abolish, or maintain at a permanently low level, the excretion of the acetone bodies. In this way it is in contrast with the other cases.

CONCENTRATION OF ACETONE BODIES IN THE BLOOD AND URINE.

Considerable interest attaches to the concentration of the acetone bodies in the blood and the rate of their excretion into the urine, as the balance between the rate of production and the rate of elimination of these acids will be a powerful factor in determining the presence or absence of an acidosis.

Reference to the tables already given shows that the concentrations of "acetone" in the blood are not remarkably high. In seven cases, only one showed a concentration as high as 0·4 gm. per litre, while all the others

varied from 0·1 to 0·2 gm. per litre. Comparisons with the "acetone" concentration in the blood in other conditions are difficult, but our figures are not to be compared with those for blood in diabetes associated with a depletion of the bicarbonate reserve of the plasma, and with the clinical manifestations of acidosis. In discussing the general results obtained on the blood "acetones" in diabetes, Van Slyke and Fitz (1917) remark that the well-managed case will show from 0·2 to 0·4 gm. per litre. Such a case of diabetes would be one in which the production of the acetone bodies had been kept sufficiently well under control so as to eliminate all immediate danger of coma, and the blood would show a normal or only slightly lowered alkaline reserve. This agrees with the findings in nausea and vomiting of pregnancy. In our own series of cases we looked for evidence of acidosis in the CO_2 content of alveolar air, using the Fridericia apparatus. With one exception (Case V) we obtained normal figures, and even in this case the value had risen to normal two days subsequent.* Losee and Van Slyke (1917) have examined the plasma bicarbonate reserve in pernicious vomiting and found normal or slightly subnormal values. There is, then, no evidence in the blood or the alveolar air that anything but the mildest disturbance is taking place in the direction of acidosis. Incidentally, one may note here that the giving of bicarbonate in vomiting of pregnancy has no justification from this standpoint.

Contrasted with the concentration in the blood, the concentration of the "acetone" in the urine is sometimes remarkably high. The daily excretion, as seen in the tables attached to the individual cases, may also be quite large in amount. The acids, then, are eliminated from the body in high concentration and large amount. Indeed, we may remark that the concentration of the total acetone bodies in three of our cases (IV, V and VII) is higher than the maximum given for diabetes. Allen, Stillman and Fitz (1919), in their studies on diabetes, came to the conclusion that the possible concentration of the acetone bodies in the urine was limited, the highest observed being between 9 and 10 gm. per litre. Whilst we would not like to insist that the maximum concentration given by Allen, Stillman and Fitz might not be exceeded in diabetes, still, it does demonstrate clearly that the concentration of the acetone bodies in vomiting of pregnancy may be extraordinarily high.

The total amounts of "acetone" excreted, too, are large, comparable in amount with those found in many cases of severe diabetes. They are distinctly higher than figures for the usual "acetone" excretion in starvation, though there are notable exceptions.

In conclusion, then, there is in vomiting of pregnancy a large production of aceto-acetic and β-oxybutyric acids, which is rapidly and promptly eliminated in the urine in high concentration, leaving the concentration in the blood at low levels. They do not accumulate in the blood to the extent found in diabetic comas, and their presence is not associated with any disturbance of the alkaline reserves. It is pertinent, therefore, to inquire into the manner in which they are eliminated, especially to see if the high ammonia coefficients observed in this condition are related to their excretion as is the case in starvation.

* The results by the Fridericia apparatus are sometimes low without a corresponding low plasma bicarbonate (Stillman, Van Slyke, Cullen and Fitz, 1917).

EXCRETION OF AMMONIA.

A great deal of attention has been paid to the excretion of ammonia in the vomiting of pregnancy, unfortunately without, at the same time, paying attention to the excretion of acid. The misleading employment of a figure known as the "ammonia coefficient," together with its only occasional estimation instead of the determination of the ammonia day by day, has led to many erroneous conclusions. We do not wish to say that a number known as the "ammonia coefficient" or the "ammonia index" may not be used if it is so desired, but its interpretation is not so simple as that of the actual number of grammes of ammonia excreted day by day.

In our cases it is obvious (Tables I–VII) that the ammonia excretion follows in a general way the excretion of acetone bodies, and that diminution or increase in urinary ammonia is usually accompanied by similar changes in the acetone excretion, especially in cases where large amounts of "acetone" are being eliminated. That complete harmony between the curves of the two bodies does not exist is not to be wondered at. Ammonia is only one of the bases which play a part in the maintenance of the acid-base equilibrium of the body, and is normally present in the urine without the occurrence of "acetone." Gilliatt and Kennaway also observed the high ammonia index to be accompanied by an increased excretion of acetone bodies, though in neither of their cases do they possess complete data on this latter excretion. In urine containing smaller amounts of "acetone," the correspondence between the two excretions would necessarily not be so complete, for, as we have just stated, normal urine always contains a certain quota of ammonia without the presence of acetone bodies. Such results are similar to those indicated in starvation in the earlier classical researches of Brugsch (1905) and of Cathcart (1907).

The publications of Henderson and Palmer (1913) and of Fitz and Van Slyke (1917) have shown that the body makes every effort it can to avoid any wastage of fixed base. It does so in two ways:

(1) By the production of ammonia at the expense of urea.

(2) By increasing the acidity of the urine.

That this first mechanism for preserving fixed base is called into play in the vomiting of pregnancy we have just shown. Unfortunately we did not determine the magnitude of the possible second factor. It is, however, well known that urines in vomiting of pregnancy are highly acid, and Gilliatt and Kennaway in their Cases I and II show the high acidity with high acetone excretion, and a much lowered acidity when the urine approaches its more normal composition, though most of their figures are percentages and not daily amounts. Consequently we readily conclude that in pernicious vomiting the body endeavours to get rid of aceto-acetic acid and β-oxybutyric acid by eliminating part of them as such in the urine. In our opinion, then, these abnormal acids are eliminated from the body by means of the two ordinary mechanisms just stated. There is thus no reason to suppose in the average that any depletion of fixed base should occur from the body's store of reserves, though doubtless occasional cases will arise in which the production of β-oxybutyric and aceto-acetic acids may be so intense that these two are drawn upon to preserve the acid-base equilibrium, and a certain amount

becomes lost to the body. We do not wish at this stage of our inquiry to discuss the mechanism of the transport of "acetone" in the blood, though in view of what we have just stated the papers of Nash and Benedict (1922), and Gamble, Ross and Tisdall (1922) form a very valuable guide.

EXCRETION OF UREA.

The earlier investigators who were impressed by the finding of a fatty degenerated liver at autopsy in cases of pernicious vomiting attempted to find metabolic evidence of this disturbance. They pointed to the raised ammonia coefficients and the lowered urea nitrogen percentages as evidence of serious hepatic lesions. The lowered urea nitrogen percentage found by Ewing and Wolf (1907) was confirmed by Losee and Van Slyke (1917). On the whole, our series (see Tables I–VII) does not show such raised ammonia indexes and such lowered urea nitrogen percentages as may be found in the work of other investigators. Our lowest figures for urea nitrogen percentages are to be found in Case IV, in which on one day the urea nitrogen excretion represented only 43·8 per cent. of the total nitrogen. In discussing these low figures it must be borne in mind that in this patient extraordinarily low amounts of total nitrogen were observed in the urine, and as the urea fraction represents the major variable fraction of the total nitrogen, any variation in this latter is at once reflected in an enhanced manner in the urea nitrogen percentage. In order, however, to show that these low values for urea are due to the conversion of either part of the urea itself, or one of the precursors of urea, we added together the urea nitrogen percentage and the ammonia index. The sum of the two in all cases gave a value which is comparable with the value found in normal individuals when excreting similar amounts of total nitrogen. The values ranged from 80 per cent. to 95 per cent. except in Case IV, where, with the extraordinarily low total nitrogen excretion, the sum total of the urea nitrogen percentage and the ammonia index was correspondingly lowered.

EXCRETION OF TOTAL NITROGEN.

The nitrogen balance in pregnancy has been a matter of considerable interest and importance. Our exact knowledge commences with the work of Bar, and from his work and the investigations of Murlin and Gammeltoft the conclusion has developed that in normal pregnancy there are at least two stages of protein metabolism (*vide* Murlin, 1917). The first occurring, in the early part of pregnancy, shows either a negative nitrogen balance, or a lowered positive balance when compared with the balance of the previous non-pregnant period; and a second stage in later pregnancy always characterised by a positive nitrogen balance. These conclusions have been developed from work on animals, mainly dogs and rabbits, but are borne out in some degree by the observations of Landsberg and Wilson on women, although actually a negative nitrogen balance in women has never been seen in early pregnancy. As the period of negative nitrogen balance in the dog corresponded closely with the development of the placenta, and as at about the point of the completion of that organ the negative balance changed to a positive one, and

as in one of his animals Bar observed a period of distaste for food and actual vomiting, he was led to the conclusion that associated with the nitrogen loss was the so-called "physiological" vomiting of pregnancy. In this conclusion he was supported by Murlin and to a certain extent by Gammeltoft, and in this connection it is of interest to note that the cases of early pregnancy studied both by Landsberg and by Wilson had shown no trace of nausea or vomiting. Indications of the existence of this negative nitrogen balance in early pregnancy had been found by observers earlier than Bar, and though their experiments were not decisive, they had been led to the conclusion that this nitrogen loss could not be prevented by feeding, in which they were later supported by Gammeltoft. The cause of this destruction of protein was very clearly defined by Murlin as due to the parasitic life of the freshly-growing ovum, which, attacking the uterine wall by means of a series of proteolytic enzymes, derived its energy and material for growth exclusively from protein. The maternal organism, at this point, was concerned solely with obtaining a means of defence against the growing invader, and the placenta was devised as an organ of control, interposed between the fœtus and the mother. At this point the relation of parasite and host changed to one of symbiosis.

In Tables I–VII we have given the total nitrogen excretion of our series of cases. It is evident at once from an inspection of the tables that under the influence of the treatment the total nitrogen excretion shows a downward tendency. Were we dealing with a simple case of starvation such a result would be at once expected, as the sparing action of carbohydrate upon protein is a well-acknowledged fact. That, however, small amounts of carbohydrate such as we administered in these cases (50 gm. to 100 gm.) should have so marked an effect upon the total nitrogen excretion is somewhat a matter of surprise. Are we to connect nausea and vomiting of pregnancy with protein destruction brought about solely by the parasitic action of the fœtus? This lack of harmony between the course of the total nitrogen excretion in our cases and the theory of "physiological" vomiting of Bar becomes even more apparent when we notice the almost complete correspondence between the excretion of total nitrogen and the excretion of ammonia. We have already designated the part played by ammonia and its relationship to the excretion of "acetone," and it would thus appear much more likely that the total nitrogen excretion, and consequently the extent of the tissue destruction which takes place in these cases, is governed by the same factor that controls the excretion of "acetone" and ammonia. The results which we have just given are borne out in part by the figures of Underhill and Rand (1910) in their Cases 1, 2 and 4. It would thus appear possible that lack of carbohydrate is responsible for the perverted fat catabolism and the tissue destruction which we see in vomiting of pregnancy, and that protein destruction takes place to provide its moiety of glucose molecules.

The work thus described would give a dominating influence to carbohydrate in vomiting of pregnancy, but again one must compare the conclusions with those derived from a study of starvation. Landergren (1903), from his studies of nitrogen metabolism in starvation, as early as 1903 expressed the belief of the "absolute value of carbohydrate." These conclusions were supported by Cathcart (1909–10), but seem to have been overlooked or have lapsed into

oblivion in later years. Similar conclusions can thus be drawn from a study of pernicious vomiting and starvation. The fundamental lack in both is carbohydrate.

CONCLUSIONS.

a. The excretion of "acetone" in the majority of cases of nausea and vomiting of pregnancy parallels the clinical condition.

b. The effect of abortion is to diminish the production of "acetone."

c. Tissue destruction in nausea and vomiting of pregnancy when under the influence of small amounts of carbohydrate is governed by the demand for further supplies of carbohydrate.

d. The results are similar to those observed in some cases of starvation, but in pregnancy the demand for carbohydrate, and consequently the disturbance following its lack, is more intense than in starvation.

The authors wish to express their thanks to Professor B. P. Watson, Obstetrician and Gynæcologist, Chief of Service, Toronto General Hospital, for his permission to utilise these and other cases; also to thank Dr. J. Gordon Gallie for a similar permission. To Dr. H. B. Van Wyck, and Dr. D. M. Low, and Miss Dulmidge, of the Toronto General Hospital, thanks are also due for the care they exercised in the conduct of the cases.

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THE RELATIVE CONCENTRATION RATIOS OF SOME CONSTITUENTS OF THE URINE.

II.—THE RELATIVE CONCENTRATION RATIOS OF UREA, CREATININE, INORGANIC PHOSPHATE AND URIC ACID.

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Received for publication February 22nd, 1923.

SEVERAL observations have been made that the various "no-threshold" bodies may be increased unequally in the urine under certain conditions, but the results, as far as concerns theories of kidney function, are often not quite conclusive, owing to the absence of definite information as to the possible variations in their amounts in the blood at the same time.

Marshall (1920) has shown that in water diuresis, although the volume of urine is largely increased, yet the output of creatinine is unchanged, while that of urea is only slightly raised.

Carr (1921) has reported similar results. The output of urea and sulphates is always increased, that of NH_4 and P_2O_5 is generally, while the output of creatinine shows no measurable alteration. The variations in the outputs of urea and sulphates are not parallel. The volume of urine always increases to a still greater extent, so that there is in all instances a decrease in the percentage amounts present.

In the diuresis following increased renal blood-flow from section of the renal nerves, Marshall and Kolls (1919) and Marshall and Crane (1921) have obtained similar results; the creatinine excretion was unaffected, the output of urea, SO_4 and P_2O_5 somewhat raised, while the volume of urine was largely increased.

Marshall's experiments on water diuresis in man were controlled by comparing the results with those obtained at the same hours of the day, and under the same conditions, except that no water was drunk. He states that the results cannot be due to plasma changes, since even after copious water drinking dilution of the blood is extremely small (Haldane and Priestley, 1916; Priestley, 1921); moreover, analyses of the plasma in dogs showed no significant changes in urea and creatinine, although chlorides fell, accompanying an increased excretion in the urine. In these experiments some time (up to 2 hours) elapsed between the drawing of the two blood samples.

In one experiment the concentration ratios of urea and creatinine (from estimations on one hour's urine and one blood sample) were different, the latter being concentrated about one and a half times more than the urea.

The results suggest that, in diuresis, assuming the amounts in the blood to remain fairly constant, the concentration ratios of creatinine, urea and phosphates should *fall*, this fall being greatest in the case of creatinine, and less marked in the case of urea and phosphates. The concentration ratio of creatinine relative to that of urea would be less, while the relationship of the ratios of phosphate and urea might be unaltered, or the former might show a diminution relative to the latter.

While this work was in progress a paper was published by Mayrs (1922); using rabbits, he injected various no-threshold bodies in pairs, and after about five minutes, when a sufficiency of urine had been collected, bled the animals to the extent of three-quarters of their blood volume, and performed the estimations on the plasma and urine. He found that SO_4, P_2O_5 and creatinine were concentrated to about the same extent and 1·5 to 2·75 times more than the urea. He suggested that the urea was reabsorbed in the tubules, possibly subserving some useful function in the organism. This conclusion was confirmed by experiments in which one kidney secreted against pressure, and in these the effect of resistance on the output of urea was greater than on that of SO_4.

Finally, it might be mentioned that there are a number of observations obtained in cases of renal disease, and from the use of drugs, which suggest that the function of the kidneys towards any one of the no-threshold bodies may be altered without a corresponding alteration in their relationship to the others, but the relative concentration ratios have not been accurately determined.

METHODS.

In this series of experiments an attempt has been made to compare the concentration in blood and urine simultaneously, of urea, creatinine and inorganic phosphate, and in a few, of uric acid in addition.

The general plan was to inject a solution of the substances about to be examined, and after a short time take a blood sample, and a further sample after a variable length of time, during which the urine was collected.

The reasons for injecting a solution of the substances whose concentration ratios were to be determined were three in number; first, a greater amount in the blood enabled smaller samples to be collected, thus obviating too great a loss of blood, while the error incidental to calculating the concentration ratio from the mean of two samples was diminished; secondly, the greater amounts present per cent. in the urine, together with the resultant diuresis, if the amounts injected were large enough, shortened the time of collection of the urine—a smaller volume being necessary for the requisite estimations. In addition, this shorter time allowed less for material alterations to occur in the quantities present per cent. in the plasma.

Finally, with higher plasma percentages, extraneous factors have less influence on the excretion rates of the substances studied (Addis and Watanabe, 1917).

The animals used have been cats: under ether, followed in later experiments by C.-E., cannulæ were inserted into a carotid, for recording blood-pressure, and into the trachea. Preparations were also made for injecting the solution into a femoral vein, and for withdrawing blood, usually from the opposite carotid artery. A catheter was inserted into the bladder by making an incision in the urethra just above the symphysis pubis. In all experiments except the first (where urethane was given) the animal was then decerebrated by trephining. No further steps in the experiment were taken for at least half an hour.

The solution was then injected, a blood sample obtained, and the collection of urine begun. When sufficient urine had been obtained a second blood sample was drawn. Sometimes the experiment was continued, further samples of blood and urine being obtained. The bladder was either emptied by pressure, or the body was ligatured, or clamped off from the base, so that the urine flowed directly into the catheter. The blood was drawn into paraffined tubes containing sodium oxalate (powdered) in the proportion of 20 mg. to 10 c.c. of blood, and centrifuged; the plasma was used for the analyses.

The analytical methods used were the same as previously described (Underhill, 1923).

RESULTS.

The results are set out in Tables I, II and III. The amounts and natures of the substances injected are given in the second column followed by the time which elapsed before the first blood sample was drawn. In the two experiments in which uric acid was injected, it was found that the alkalinity of the Na_2HPO_4 was not great enough to dissolve it completely without the addition of a little NaOH.

During the following period urine was collected, and the total volume obtained and the times of collection are given in the next columns. Then follow the results of the analyses of the plasma and urine; in the case of the former the results of the two analyses are given, each pair of figures thus representing the amounts present in the plasma at the beginning and end of the period during which the urine was collected.

The amount in the plasma for calculating the concentration ratio is taken as the mean of the two analyses. In the last column, or columns, the ratio is compared with that of urea, taking the urea concentration ratio as=1.

In the case of the first two experiments the analyses were performed on whole blood instead of plasma, while in the first the animal was under urethane and not decerebrated.

It will be noticed on examination of the Tables that there is a general tendency for the concentration ratio to vary in the course of a single experiment, in the same direction as the concentration of the substance in question in the *urine*. This is explicable on the assumption that the concentration in the urine depends in part on the rate at which the urine is secreted: a more rapid secretion allows less time for water to be reabsorbed in the tubules, and the concentration in the urine falls and with it the concentration ratio. On the other hand, an inverse relationship between the

TABLE 1. (PART 2)

EXPERIMENT No	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL OF URINE IN C.C.	TIME OF COLLECTION OF URINE IN MINS.	UREA			CREATININE			$\frac{C}{Uv}$
					PLASMA* mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = Uv	PLASMA* mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = C	
1† (MALE) 3·5 K	UREA 0·12 g CREATININE 0·005 g IN 5 c.c. SALINE	4·4	5·0	51	51·6 55·2	726	13·6	1·3 1·3	4·3	3·3	2·4
2 (MALE) 3·2 K	(a) UREA 0·05 g CREATININE 0·005 g IN 25 c.c. SALINE	7	5·7	39	43·1 41·7	964	22·7	1·4 1·3	59	43·7	1·9
	(b) UREA 0·02 g CREATININE 0·002 g IN 10 c.c. SALINE	2	7·9	42	50·4 61·9	412	7·3	1·5 1·4	29·5	20·3	2·8
8 (MALE) 3·5 K	UREA 0·25 g CREATININE 0·01 g IN 5 c.c. SALINE 50 c.c. SALINE	49 5	1·95	43	72·0 71·3	2775	38·7	1·7 1·6	25·4	15·4	4·0
9 (MALE) 2·75 K	UREA 1·0 g CREATININE 0·05 g IN 50 c.c. SALINE	2	3·6	26	157·0 105·0	3644	27·8	8·2 3·0	47·0	84·0	3·0
11 (MALE) 4·3 K	UREA 1·09 CREATININE 0·05 g IN 50 c.c. SALINE	2	(a) 1·9	15	141·0 95·0	947	8·0	5·1 3·7	9·0	18·4	2·3
			(b) 1·6	20	95·0 104·0	1193	12·0	3·7 3·4	16·0	45·0	3·7

† CAT UNDER URETHANE.
* IN EXPERIMENTS Nº 1 & Nº 2 THE ANALYSES WERE PERFORMED ON WHOLE BLOOD

TABLE 2. (PART 2)

EXPERIMENT No	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL OF URINE IN C.C.	TIME OF COLLECTION OF URINE IN MINS	UREA			CREATININE			INORGANIC PHOSPHORUS			URIC ACID			$\frac{C}{Uv}$	$\frac{P}{Uv}$	$\frac{UA}{Uv}$
					PLASMA: mg PER 100 c.c.	URINE: mg PER 100 c.c.	CONCENTRATION = Uv	PLASMA: mg PER 100 c.c.	URINE: mg PER 100 c.c.	CONCENTRATION = C	PLASMA: mg PER 100 c.c.	URINE: mg PER 100 c.c.	CONCENTRATION = P	PLASMA: mg PER 100 c.c.	URINE: mg PER 100 c.c.	CONCENTRATION = UA			
12 (MALE) 3·64 K	UREA 1·0 g CREATININE 0·05 g $Na_2HPO_4, 12H_2O$ 0·5 g URIC ACID 0·025 g IN 12·5 c.c. DISTILLED WATER	10	3·15	20	117 101	2400	22	3·9 3·2	195	55	12·6 8·3	253	24	0·65 0·53	50·6	86	2·5	1·1	3·9
	50 c.c. SALINE	7																	
13 (MALE) 4·0 K	UREA 1·0 g CREATININE 0·05 g $Na_2HPO_4\,12H_2O$ 0·5 g URIC ACID 0·25 g IN 27·5 c.c. DISTILLED WATER	34	(a) 2·8	10	73·7 92·5	989	12	1·9 2·4	92	43	7·7 11·5	109	11·0	3·6 3·0	122	37	3·6	0·95	3·1
			(b) 4·45	13	92·5 101·0	728	7·5	2·4 2·4	63	26	11·5 8·9	90	9·0	3·0 1·7	69	29	3·5	1·2	3·9
			(c) 4·62	15	101·0 103·2	759	7·4	2·4 2·4	58·5	24	8·9 8·5	72	8·0	1·7 1·1	50	36	3·3	1·1	4·8

concentration in the urine and the concentration ratio may occur: in these instances an explanation must be sought in the amounts available for excretion, as indicated by the behaviour of the concentration in the plasma.

The creatinine is always concentrated to a greater extent than the urea—from two to five times more in the different experiments. From experiments 11 and 13 it will be seen that the relative concentration ratios follow the movements of the urinary concentrations of urea and creatinine. This can be explained on the basis of the filtration reabsorption theory; as the passage of the urine along the tubules becomes slower, so there is more time for water to be absorbed and with it some of the urea, if this modification of the theory be accepted; and the reverse occurs when the concentrations in the urine fall.

TABLE 3. (PART 2)

EXPERIMENT No	INJECTION	PERIOD BETWEEN INJECTION & FIRST BLOOD SAMPLE IN MINS.	VOL. OF URINE IN C.C.	TIME OF COLLECTION OF URINE IN MINS.	UREA			CREATININE			INORGANIC PHOSPHORUS			C/Uv	P/Uv
					PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = Uv	PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = C	PLASMA mg PER 100 c.c.	URINE mg PER 100 c.c.	CONCENTRATION = P		
16 (MALE) 4·1K	UREA 1·0g CREATININE 0·1g $Na_2H_2PO_4$, H_2O, 1·0g IN 20 c.c. DISTILLED WATER	10	(a) 3·3	30	120 103	842	7·5	9·7 5·7	260	33·8	33·7 22·6	409	14·5	4·5	1·9
			(b) 2·6	15	103 106	936	9·0	5·7 4·8	242	46·1	22·6 18·0	456	22·5	5·1	2·5
			(c) 1·65	30	106 117	990	8·9	4·8 4·9	223	46·0	18·0 17·9	438	24·4	5·2	2·7
17 (FEMALE) 2·75 K	UREA 1·0g CREATININE 0·1g $Na_2HPO_4, 12H_2O$ 2·5g IN 25 c.c. DISTILLED WATER	8	(a) 5·8	10	125 95	768	7·0	11·9 7·3	140	14·6	49·3 35·2	466	11·0	2·1	1·6
			(b) 3·9	12	95 105	763	7·6	7·3 6·2	146	21·6	35·2 28·0	486	15·4	2·8	2·0
			(c) 4·4	10	105 97	849	8·4	6·2 5·3	126	21·9	28·0 25·4	452	16·9	2·6	2·0

In experiments 16 and 17 the concentrations of urea and creatinine in the urine vary in opposite directions, with a tendency for the relative concentration ratio to follow the urea concentration: variations from this relationship possibly depend on the fact that the plasma concentrations of the two substances do not always vary in the same direction.

In experiment 2, while there is a fall in the urinary concentrations, yet there is a rise in the relative concentration ratio, and this is accompanied by a rise in the concentration of urea in the blood. It is possible that the fall in urinary concentrations indicates a lack of appreciable excess to be excreted rather than a diuresis; only very small quantities had been injected previously. These results, then, agree in most instances with the conclusions deduced from the experiments of Marshall and Kolls (1919) and Marshall and Crane (1921).

The general results of my experiments agree, in spite of the fact that the plasma concentrations may vary in the same or in opposite directions,

or remain almost constant. Under the conditions of the experiment it is considered that where a substance shows an almost constant plasma concentration as estimated from the two samples, there has been no appreciable fluctuation of the percentage amount in the plasma during the time of collection of the urine, and that the concentration ratio represents fairly accurately the relation between the plasma and urinary concentrations. The only other source of error to be considered is the possibility that the urine collected may not accurately represent the urine secreted during the time between the drawing of the two samples. The urine contained in the catheter was always allowed for, and the bladder was always emptied, so that only the urine in the tubules of the kidney and ureter need be considered: in the cat's kidneys this possibly amounts to about 0·2 c.c. (calculated from figures given by Cushny, 1917). However, this error is presumably not great, considering the similarity of the results obtained with different rates of secretion of urine; in cases where a diuresis occurred the error becomes negligible.

The fact that the results obtained when the plasma concentrations are constant are comparable with those obtained when these concentrations varied, suggests that taking the mean plasma concentration for calculating the concentration ratio introduces no appreciable error, the concentration in the plasma falling or rising, more or less rapidly, from the first to the second figure, without any intermediate considerable fluctuations.

In those experiments in which the plasma concentration remained almost constant, the excretion of substances in the urine must be balanced by a corresponding absorption from the tissues, or by a concentration of the blood. Attempts have been made to follow the latter by means of hæmoglobin estimations. After the dilution following the injection the concentration remains almost constant, usually with a tendency to increase during the course of the experiment.

Turning now to the results reached with inorganic phosphate and uric acid, it is not wished to lay great stress on those obtained with the latter, except to point out that the degree of concentration compared with that of urea is of the order of that of the creatinine. Since, in the cat, uric acid is not an end-product of metabolism, but is further converted into allantoin (Jones, 1920), its disappearance from the blood is much more rapid than in the case of the other substances investigated, giving a false view of the amount to which it is concentrated in the urine relative to its plasma percentage. In experiment 13 (*a*), however, the plasma concentration only fell from 3·6 to 3·0 mg. per 100 c.c. in ten minutes, and in that period its concentration ratio relative to that of urea was 3·1, while that of creatinine was 3·6. The results, as far as they go, do not suggest that uric acid is other than a no-threshold substance in the cat.

When the disodium phosphate was injected in experiments 12 and 13, the results showed a concentration ratio for inorganic phosphorus of about the same value as that of urea; further experiments, however, gave a figure which was intermediate between that of urea and creatinine. In experiment 16 the acid sodium phosphate was used in much greater amount than the alkaline in Nos. 12 and 13—the content in phosphorus being about five times greater. It was possible that the difference in the results was due to the use of the acid

instead of the alkaline phosphate, since Nagayama (1920) found the urea-excreting power of the kidneys in rabbits less after the administration of the acid phosphate, as compared with a neutral phosphate mixture of the same phosphorus content, both being accompanied by the same amount of urea. However, experiment 17, in which the phosphorus content of the alkaline phosphate injected was about equal to that of the acid phosphate of experiment 16, suggests that the results may be explained by considering the amounts of the various substances the kidneys have to remove from the body: with only a small amount of phosphate injected the concentration ratio approaches that of urea; with a larger amount the kidney removes it with greater ease and in higher concentration than the urea; in both cases it is concentrated to a smaller extent than the creatinine. Nagayama (1920) found the urea excretion in rabbits to be more effective if phosphate was given with the urea, but this was perhaps in part due to a greater diuresis.

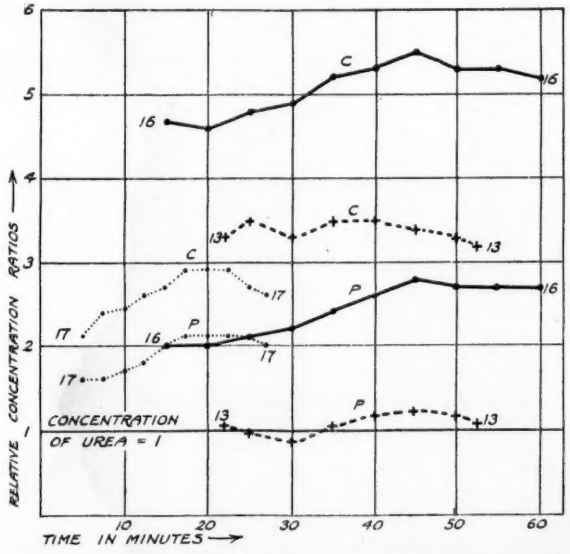

FIG. 1.—C = Creatinine. P = Inorganic phosphorus. The curves were obtained as follows: In each of the three experiments the concentrations of the different blood and urine samples were plotted along the ordinate against time of collection along the abscissa. The urine concentrations were plotted at points mid-way between the time of withdrawal of the corresponding blood-samples. Intermediate values at successive intervals of time were then read off, and the concentration ratios calculated. The values obtained are expressed relative to the concentration ratio of urea, taken as = 1. Zero time is the moment when the injection was made into the blood-stream.

It is possible that with still larger injections of phosphate the concentration ratio would approach yet closer to that of creatinine, as Mayrs (1922) found.

In Fig. 1 are plotted the concentration ratios of creatinine and inorganic

phosphate relative to that of urea, which is taken as = 1, in the three experiments, 13, 16 and 17. The figure shows the varying degrees to which the three substances are concentrated; it also makes clear a general parallelism between the ratios of creatinine and inorganic phosphate, which probably depends on the rate of blood-flow through the kidney.

The fact that urea, inorganic phosphate and creatinine may be concentrated to different degrees by the kidneys during excretion is difficult of explanation on the filtration reabsorption hypothesis. It seems possible that the kidneys secrete them independently according to the requirements of the moment; and yet a fairly definite relationship between their excretion rates was clear in all experiments in which several samples of urine were obtained (*v.* Table IV). It will be seen that the amounts excreted, expressed as mg. per hour, usually vary in the same direction in the different samples of a single experiment, although not exactly in equal proportions. This factor correlating the excretion rates is presumably the blood-flow through the kidney. Attempts were made to correlate the variations with variations of blood-pressure, but the relationship was by no means definite.

It was thought that some direct evidence might be thrown on the question whether urea has a " threshold," and so might be expected to be re-absorbed in the tubules, by experiments in which the formation of urea was cut down as far as possible and the amount in the blood estimated after some time. For this purpose some observations were made on animals which had been eviscerated before decerebration; the liver was left in the body, but with the structures at the portal fissure occluded by a ligature. In no case, however, was a definite result reached, the animal dying before the level of urea in the blood had fallen to any great extent. It is doubtful if this direct method of attack will be successful, since urea is possibly formed in other parts of the body than the liver (Folin and Berglund, 1922).

TABLE IV.—*Excretion Rates in Successive Samples of Urine (expressed as mg. per hour).*

Experiment No.	Urea.	Creatinine.	Inorganic phosphorus.
2	84·5	5·2	—
	46·5	3·3	—
11	72·0	6·8	—
	57·0	7·6	—
13	60·8	5·2	6·8
	166·2	15·6	18·0
	150·0	13·1	18·5
	140·0	10·8	13·2
16	55·6	17·2	27·0
	97·2	25·2	47·6
	32·6	7·4	14·4
17	267·0	48·6	162·0
	149·0	28·5	94·5
	224·4	33·0	119·4

Baglioni (1905, 1917) and Fühner (1908) have shown that urea is necessary for the proper working of the excised perfused selachian heart: it is possible that urea performs a useful function in the body of the mammal also. The experiments on the relative concentration ratios, however, lend no support to this view, as far as they bear on the question of the re-absorption of urea in the tubules of the kidney.

It was of interest to determine the rate at which the injected substances left the blood-stream: this was done by taking the blood volume as 5 per cent. of the body-weight and calculating the total amounts of the various substances in the blood before injection. Where a sample of blood was not drawn before injection, average normal values for the concentrations in the blood were taken as the basis of the calculation.

From these figures, together with the percentage amounts present in the blood after the injection, the quantity of injected substance still in the blood within a few minutes after injection could be roughly computed: the remainder must be in the tissues. Some time after the injection allowance must be made for the amounts excreted in the urine; if a large quantity of fluid was injected, an allowance could also be made for this addition to the blood volume.

The results show that within a couple of minutes after injection from 75 to 90 per cent. of the injected urea and creatinine are no longer to be found in the blood-stream: Marshall and Davis (1914) found a similar disappearance from the blood-stream on injecting large amounts of urea into dogs. Subsequently there is a further small loss to the tissues. The figures indicate that these substances leave the blood very rapidly at first, and that the rate then slows down until a balance is struck between the concentrations in the blood and in the tissues. Urea at any rate seems to be evenly distributed in the blood and the tissues throughout the body, except for the fat, where there is extremely little (Karr and Lewis, 1916; Gad Andresen, 1921). Reimann and Sauter (1921) also observed that $NaHCO_3$ injected into the dog is rapidly distributed between the blood and lymph.

The phosphate seemed to behave differently from the urea and creatinine, in that a distinctly smaller proportion left the blood-stream for the tissues, the figures varying between 65 and 80 per cent. of the total injected. This difference in their behaviour is still seen, even when allowance is made for the fact that the phosphate tends to be more concentrated in the plasma, as compared with the corpuscles, so that estimations made from plasma analyses tend to show too high a proportion of the injected substance remaining in the blood-stream.

SUMMARY.

1. In the cat the kidneys may concentrate urea, creatinine and inorganic phosphate to varying and unequal extents. The creatinine is concentrated from two to five times more than the urea; the inorganic phosphate may be concentrated to about the same extent as the urea, or up to nearly three times more. The creatinine and inorganic phosphate are not concentrated to the same degree, the former being always more concentrated by the kidney during excretion than the latter.

2. The excretion rates of urea, creatinine and inorganic phosphate show a general relationship, rising and falling together though not to the same relative degrees. The correlating factor is probably the blood-flow through the kidney.

3. After injection, urea and creatinine leave the blood-stream extremely rapidly, 90 per cent. of that injected being in the tissues within two to three minutes after completion of the injection. The rate of disappearance of inorganic phosphate is not quite so rapid, and a greater proportion remains in the blood as compared with that passing into the tissues.

4. It is considered that the results of the experiments on the relative concentration ratios of urea, creatinine and inorganic phosphate are incompatible with the "filtration reabsorption" hypothesis of kidney function in its present form. It is concluded that the kidneys must actively secrete one or more of these substances into the urine.

I wish to thank Prof. C. Lovatt Evans for help and advice during the course of this investigation.

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A METHOD OF ESTIMATION OF DIASTASE IN BLOOD.*

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Received for publication March 22nd, 1923.

THERE is a general acceptance of the view that estimation of blood diastase is of distinct diagnostic value, and it is unfortunate that certain features of recent methods render them not entirely satisfactory. Stocks (1916), and later Harrison and Lawrence (1923), adopting Wohlgemuth's method, incubate a series of dilutions of blood serum, or blood plasma, with starch and obtain their results from the colour reaction which occurs on the addition of iodine. In the first place it is very difficult to obtain in general practice a sufficiency of serum for the purpose of the test (from 1 c.c. to 3 c.c.) without veni-puncture, and, in the second place, sera do occur occasionally of which the tint definitely masks the delicacy of the colour reaction. Myers and Killian (1917), using the Lewis and Benedict method of blood sugar estimation, determine the amount of sugar formed from a known quantity of starch by 2 c.c. blood after incubation for a definite period, calculating their results in terms of the percentage of starch reduced. Here again the question of veni-puncture arises, and in addition that of the inaccuracies which the picric acid methods of sugar estimation present as compared with more recent means of blood-sugar determination.

In view of these difficulties the problem of the estimation of blood diastase was approached, and, by an adaptation of MacLean's (1919) method of blood-sugar estimation, a means has been evolved which has the advantage of simplicity and accuracy, while it necessitates the minimum of discomfort to the patient. It also permits of an almost concurrent reading of the blood-sugar and the blood-diastase figures, dispensing with the need for two different techniques. For the method proposed 0·2 c.c. blood only is required—an amount which can easily be obtained by pricking the ear or the finger. The final result depends upon a determination of the patient's blood sugar by MacLean's method, and upon a second determination after 0·2 c.c. of the patient's blood has been incubated for half an hour at 37°C. with 1 c.c. of a 0·1 per cent. solution of starch.

The Effect on Blood Sugar of Incubation at 37° C.

In view of the fact that the literature of the subject gives most discordant views on the problem of glycolysis in blood, it was thought best to make an investigation of the question. It was found that under the experimental conditions described below no glycolysis was in evidence. Freshly drawn blood to the amount of 0·2 c.c. was pipetted into a small Erlenmeyer flask containing 2·8 c.c. normal saline solution. The suspension was incubated for half an hour in a water-bath at an accurately maintained temperature of 37°C. During this period a direct control estimation of the sugar in another sample of 0·2 c.c. was done. When the incubation time was completed the amount of sugar

* Work carried out in the tenure of a Carnegie Research Assistantship.

present in the specimen was estimated. Table I shows the results in six cases taken from some forty.

TABLE I.—*The Effect of Incubation on the Sugar in 0·2 c.c. Blood.*

Case.	Control percentage.	Percentage after incubation.
1	·081	·082
2	·097	·097
3	·102	·106
4	·13	·13
5	·097	·097
6	·131	·134

No appreciable change in the sugar content occurs on incubation of 0·2 c.c. blood in 2·8 c.c. saline solution at 37° C. for half an hour. It should be stated here that throughout the experiment and also throughout the entire diastase investigation, strict aseptic precautions were observed to exclude the possibility of bacterial action during incubation.

THE PROPOSED METHOD DESCRIBED.

Into one of two 100 c.c. Erlenmeyer flasks 1·8 c.c. of 0·9 per cent. saline solution and 1 c.c. of 0·1 per cent. starch solution (*i. e.*, 1 mg. starch) is accurately pipetted, while into the other (control flask) exactly 2·8 c.c. of 0·9 per cent. saline solution is introduced. 0·2 c.c. blood is withdrawn from the finger into a special MacLean pipette* and carefully ejected *into* the fluid of the first flask, the point of the pipette being held beneath the surface of the solution, while the flask is held at an angle. The pipette is rendered free from blood by repeated washing with the clear fluid into which the blood has just been delivered. The flask is then gently shaken with a circular movement so as to mix thoroughly the blood and the solution. A second sample of 0·2 c.c. blood is similarly delivered and washed into the control flask. Both flasks, provided with rubber stoppers, fitted with capillary points, are placed in a water-bath, the temperature of which is accurately maintained at 37° C. Incubation is allowed to proceed for exactly half an hour, at the end of which time the flasks are removed and 21 c.c. of MacLean's acid sodium sulphate solution is added. In the case of the first flask the addition should be made immediately on removal from the water-bath so as to stop the action of the diastase. The subsequent steps in the estimation are precisely as described by MacLean. Briefly the treatment is as follows: The flasks are heated till the boiling-point is just reached. 1 c.c. dialysed iron is added to each and after cooling under the water tap the contents are filtered. To 20 c.c. of each filtrate is added 2 c.c. alkaline copper solution. The resulting solutions are then boiled for six minutes over a flame suitably adjusted to effect distinct boiling in one minute forty seconds. At the end of that period the flasks are immediately plunged into cold water and cooled thoroughly. 2 c.c. of 75 per cent. HCl (or H_2SO_4) are added, and after effervescence has finished and after standing for one minute with occasional agitation, the iodine content of the solutions is found by titration with N/400 sodium thiosulphate. During titration, in the case of the first flask, a variation of colour is seen ranging from dark amber to

* To be obtained from Hawksley & Son, Wigmore St., London.

leaden and to pale blue, the final disappearance of which marks the completion of titration; in some cases when the blue colour is very faint the end-point may be rendered more distinct by the addition of a drop of 3 per cent. starch solution. The amount of sodium thiosulphate used is noted and its exact glucose equivalent ascertained from the glucose-thiosulphate table, or very much better, from a plotted graph. The second flask is treated in exactly the same manner except that starch must be added to complete the titration.

Calculation.

The result is calculated as the percentage of soluble starch transformed to sugar (calculated as glucose) by the 0·2 c.c. blood employed. The amount of starch used is 1 mg., and the difference between the sugar contents, measured in fractions of a milligramme, of the two samples of 0·2 c.c. blood will be equivalent to the amount of starch reduced to sugar.

Suppose that, reading from the glucose-thiosulphate graph, the sugar content of the control preparation is 0·164 mg., while for the starch preparation it is 0·259 mg. Since the amount of filtrate used corresponds to $\frac{4}{5}$ of 0·2 c.c. blood, that amount of blood would contain 0·205 mg. sugar in the one case and 0·323 mg. sugar in the other. The difference, 0·118, is equivalent to the amount of starch transformed to sugar by incubation with 0·2 c.c. blood, so that the diastatic index in this case is 11·8. Allowance should, of course, be made for any slight reducing action of the soluble starch. During the course of this investigation the starch solutions were repeatedly tested for any such action, and in no case was it found necessary to make correction.

In view of the fact that glycolysis does not occur on incubation of 0·2 c.c. blood under the conditions of the experiment, there appears to be no good reason for running a control. The half hour during which the starch preparation is incubating may be very conveniently occupied in making a direct estimation of freshly-drawn blood.

DETAILS OF METHOD AND RESULTS.

Accuracy.—It may seem superfluous to call attention to the need for accuracy in this estimation, but in view of the fact that determinations are made in milligrammes and fractions of milligrammes, perhaps a few points which lend themselves to precision are worthy of note. Cleanliness and attention to aseptic precautions should be maintained throughout. Burettes, flasks and pipettes should be thoroughly cleaned. Antiformin or a solution of potassium bichromate in sulphuric acid is a very useful help. Standardised burettes and pipettes should be used. During the process of the boiling of the blood filtrate and the copper solution, the manometer should be carefully watched for any change in gas pressure and any necessary adjustment made.

Preparation of the starch solution.—Lintner's soluble starch was used throughout this investigation. The solvent was physiological salt solution prepared with water, double (glass) distilled and practically neutral to rosolic acid. The presence of the salt solution prevents hæmolysis, and tends to accelerate the action of diastase. A litre of 0·9 per cent. NaCl is prepared with re-distilled water, and 0·2 grm. of soluble starch weighed out and suspended in about 5 c.c. of the saline solution. Sufficient saline to make 200 c.c. is measured out and heated in a large flask almost to boiling-point,

when the starch suspension is carefully added drop by drop, the contents of the flask being shaken after each addition. A reflux condenser is then attached, and the solution allowed to boil for about ten minutes. The resulting starch solution is homogeneous and transparent, and allows of an intimate association of the enzyme and substrate. It is not advisable to submit the small quantity of starch to prolonged boiling, as is recommended by certain authors (Sherman, Kendal and Clark, 1910), who, however, use a larger quantity of starch. Table II shows how the diastatic index tends to be lowered when starch which has been boiled for some time is used in the test.

TABLE II.—*Showing Variations in Diastatic Activity when 0·2 c.c. Blood is treated with Starch Solutions boiled for Different Lengths of Time.*

Case.	Starch solution boiled for— ½ hour.	1 hour.	2 hours.
	Reducing sugar formed.		
1	·251 mg.	·230 mg.	·200 mg.
2	·180 ,,	·145 ,,	·127 ,,
3	·230 ,,	·165 ,,	·16 ,,
4	·108 ,,	·098 ,,	·08 ,,
5	·143 ,,	— ,,	·035 ,,

The starch solution should be made with great care, and should be freshly prepared every second or third day. No advantage seems to be gained in the use of glycogen instead of starch (Table III).

TABLE III.—*Showing Comparative Activity of Diastase on Starch and Glycogen.*

Case.	Blood sugar.	Sugar formed. 0·1 per cent. starch.	0·1 per cent. glycogen.
	per cent.	mg.	mg.
O. B—	·13	·200	·130
H. M—	·10	·087	·08
E. A—	·11	·141	·14
A. T—	·09	·093	·083
A. I—	·195	·315	·320

The diastatic figures of six normal subjects are given in Table IV. The figure seems to vary in different individuals, the average being about 8 to 10—a figure somewhat lower than that of Myers and Killian (1917), and similar to that of Stocks (1916). From data obtained it would seem that the normal index should not exceed 15.

TABLE IV.—*Showing the Diastatic Activity of Blood in Normal Cases.*

Case.	Age.	Sex.	Diastase.	Blood sugar.
R. R—	30	M.	8·5	·102 per cent.
E. A—	22	M.	9·0	·097 ,,
A. T—	33	M.	11·8	·134 ,,
A. G—	34	M.	8·8	·102 ,,
C. R—	30	M.	7·1	·131 ,,
W. S—	30	M.	6·5	·106 ,,

Table V gives the diastatic activity of three cases of diabetes. In the case D. G—, cardiac complications and pulmonary fibrosis were present.

TABLE V.—*The Diastatic Activity of the Blood in three Abnormal Cases.*

Case.	Age.	Sex.	Date.	Blood diastase.	Blood sugar per cent.	Urine diastase.	Urine sugar per cent.	Diet.	Remarks.
A. I—	39	M.	13/12/22	24·5	·19	—	·5	Restricted	—
D. G—	28	M.	16/2/23	14·5	·111	6·6	·25	Not restricted	No albumin in urine.
A. C—	30	M.	8/3/23	2·375 3·125	·122 ·111	10 12	·66 ·4	Restricted	No albumin in urine.

In the course of this investigation certain points have arisen which seem to suggest that the hourly diastatic activity of the blood should not be represented by a constant figure as has been suggested by some. An investigation of the matter has already been commenced and certain observations have been made.

ADVANTAGES OF THE PROPOSED METHOD.

Sugar estimations with picric acid very frequently give results which are decidedly too high. This may be due to—

(*a*) The disturbing effect of creatinine, which varies in amount pathologically and physiologically. In certain renal conditions creatinine is found in excess. On ingestion of food it may be increased, as has been confirmed by many observers, in particular Rose and Dimmitt (1916), who showed that the excess may continue for some days after ingestion. On muscular exercise an increase may be found if measured at a short interval after exercise (Schulz, 1912). No doubt other metabolic influences may have an effect on the creatinine content.

(*b*) The presence of an unknown substance or substances in the red blood-cells, as demonstrated by De Wesselow (1919), who found marked variation in the sugar concentrations depending on the number of red blood-corpuscles, and obviously not due to creatinine.

The present method has the advantage of avoiding these sources of inaccuracy. In addition, its simplicity, rapidity and cheapness are notable considerations; the avoidance of the necessity of using such expensive apparatus as a colorimeter, as in Benedict's and Folin's methods, is important.*

I am indebted to Prof. J. A. MacWilliam and to Prof. Hugh MacLean for much kindly help and criticism.

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* The necessary equipment for the present method can be obtained from Hawksley & Sons, Wigmore Street, London, for about three pounds.

THE PRECIPITATION OF COLLOIDAL GOLD BY CEREBRO-SPINAL FLUID; THE DIAGNOSIS OF NEURO-SYPHILIS.

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Received for publication April 9th, 1923.

THE use of colloidal gold for the diagnosis of neuro-syphilis has not come into general use because of the difficulty of preparing a standard colloidal gold solution and the apparent unreliability of the test. In this paper we describe a new and simple method of preparing gold and specify in detail the various precautions to be adopted in the process. We also describe the conditions under which colloidal gold is precipitated by normal and pathological cerebrospinal fluids. Finally we discuss the specific mechanisms which underlie the reactions observed in the precipitation of colloidal gold by cerebrospinal fluids obtained from cases of neuro-syphilis.

The precipitation of colloidal gold by cerebrospinal fluid was first investigated by Carl Lange (1912). He found that colloidal gold was not precipitated by cerebrospinal fluids obtained from normal individuals, but that cerebrospinal fluids obtained from people suffering from certain affections of the central nervous system gave definite precipitation reactions. He observed that these precipitations invariably occurred with cerebrospinal fluids obtained from luetic affections of the central nervous system, and considered that the colloidal gold test was of great value in the diagnosis of these conditions. These observations of Lange have been extensively investigated by various observers and have been substantially confirmed.

In 1914 Miller and Levy (1914) emphasised the fact that the precipitations of colloidal gold by cerebrospinal fluids from cases of tabes and general paresis differ in a fundamental manner, and that the gold test could be used not only to differentiate luetic from non-luetic conditions, but also to distinguish tabes from general paresis. Miller, Brush, Hammers and Felton (1915) confirmed the observations of Miller and Levy. They concluded that the reaction is specific in cases of general paresis, but not absolutely characteristic of tabes or cerebrospinal syphilis. They also observed that the paretic

* The expenses of this work were paid for by a grant from the Medical Research Council to T. A.-D.

curve was occasionally given by cerebrospinal fluid obtained from syphilitic patients showing no evidence of dementia. Similarly, Swalm and Mann (1915) found a typical reaction in 90 per cent. of cases of paresis, but doubted whether the reaction is entirely specific. Hammes (1917), however, regards the test as more delicate than any other as an index of pathological change in the cerebrospinal fluid. He finds a typical luetic precipitation may be obtained from cerebrospinal fluid giving a negative Wassermann, but absence of precipitation of colloidal gold by cerebrospinal fluid has never been obtained with fluids giving a positive reaction.*

The mechanism of the reaction between the colloidal gold and the cerebrospinal fluid has been the subject of many investigations. Lange assumed that the reactions were due to the production in the cerebrospinal fluid of specific proteins. Weston (1918) found that the gold precipitating substance is not the Wassermann-producing substance and can be separated from it. He assumed that the active precipitating agent in cerebrospinal fluid is a globulin since it is precipitated from solution by ammonium sulphate and is destroyed by heat. In this respect it is necessary to observe that Weston did not consider an increase in globulin alone indicative of syphilis, since such an increase is found in the cerebrospinal fluids of many patients not suffering from syphilis. Felton (1917) considered that the various types of reaction were due to the antagonistic precipitating relations of albumen and globulin, the albumen exerting an inhibitory action and the globulin a precipitating action. This hypothesis was put forward independently by Cruickshank (1920). Cruickshank made a detailed investigation into the value and mechanism of the colloidal gold test. He confirmed the conclusions of Weston that the active substance in the spinal fluid of general paretics is not dialysable and resides in the globulin fraction of the protein, but stated that it is not altered by heating to the coagulable point of protein. He observed that the various types of luetic reactions could be simulated by mixtures of globulin and albumen, the globulin acting as a precipitating agent and the albumen as a protective agent. He therefore assumed that the syphilitic reactions are in part due to the presence of albumen sufficient in quantity to partially obscure the precipitating effect of globulin, and in part to a specific alteration in the physical state of the globulin which is associated with a positive electrical charge.

PREPARATION OF COLLOIDAL GOLD.

A large number of methods of preparing colloidal gold have been described by different observers, and for each of these methods particular advantages and ease of preparation are claimed. All the methods, except that described by Black, Rosenberg and McBride (1917), fail in one essential detail—they give no definite directions for controlling the reaction of the gold solution obtained. We shall show in the following pages that a variation in the reaction of a gold solution to the extent of one thousandth normal determines whether a particular sample of cerebrospinal fluid reacts as a paretic or normal fluid.

* Extensive references to this question are given in the papers by Miller, Brush, Hammers and Felton, and by Cruickshank (1920).

In the method which we have devised only neutral substances are used, and the gold solutions obtained, are, in every respect, remarkably uniform.

Reagents required.—(1) Freshly-distilled water obtained by re-distilling laboratory-distilled water immediately before use. The distilling flask, condenser and collecting flask should be made of hard glass.

(2) 1 per cent. gold chloride solution prepared by dissolving a 15-grain tube of gold chloride in 100 c.c. of distilled water.

(3) 1 per cent. potassium oxalate solution prepared by dissolving 1 gramme of neutral potassium oxalate in 100 c.c. of distilled water.

Method.—To 100 c.c. of distilled water 1 c.c. of the potassium oxalate solution is added and the fluid is heated to boiling-point. Whilst boiling, 1 c.c. of the gold chloride solution is added drop by drop. Within half a minute a magnificent red solution of colloidal gold is obtained.

Precautions.—(1) The water should be freshly distilled. Water left in the laboratory for 24 hours gives a gold solution of a different degree of sensitivity to that obtained from freshly distilled water (distilled water used after a 2-days' fog gave a gold solution which precipitated spontaneously). The necessity for using freshly-distilled water depends upon the fact that traces of acid from the atmosphere (carbonic or other acids) very markedly effect the aggregation of the colloidal gold particles. The gold solution contains only one part of gold in ten thousand parts of water, so that a minute percentage of acid in the water attains a high value when compared in molecular magnitude to the gold.

(2) No special precautions require to be taken with the gold chloride or potassium oxalate solutions. Stock solutions appear to keep uniform over periods of months. Further, the exact amount of potassium oxalate used is not of great significance. Quantities of potassium oxalate solution varying from 0·8 c.c. to 1·2 c.c. per 100 c.c. of distilled water do not affect the sensitivity of the resulting gold solution. There is no advantage to be gained by increasing the quantity of gold chloride beyond 1 c.c. of a 1 per cent. solution to each 100 c.c. of water. A gold sol of maximum colour intensity is obtained by using this quantity.

(3) All the glassware used should be clean and of a hard variety, especially the glass beaker in which the gold sol is prepared. It is obvious that during the process of boiling any soluble alkali in the glass will tend to pass into solution. Alkali is just as detrimental as acid to the preparation of an adequate gold sol.

Testing of cerebrospinal fluid.—Throughout the investigation the methods and nomenclature of Miller and Levy were used. 1 c.c. of 0·4 per cent. NaCl was put into each of a series of ten test-tubes. To the first tube an additional 0·8 c.c. of 0·4 per cent. NaCl was added and also 0·2 c.c. of the cerebrospinal fluid, thus making the total volume 2 c.c. After mixing, 1 c.c. of this solution was added to the second tube, thus bringing the volume of fluid in that tube to 2 c.c. After mixing, 1 c.c. of this fluid was transferred to the third tube and so on. In this way a series of (n) tubes containing 1 c.c. of approximately 0·4 per cent. NaCl and quantities of cerebrospinal fluid varying from 1/10 c.c. to $1/10 \times 2^{n-1}$ c.c. were obtained. To each of these tubes 5 c.c. of colloidal gold was added and after 24 hours the precipitation was noted. The degree

of precipitation varied and was designated by the numbers 0 to 5, "0" implying no precipitation and "5" complete precipitation. Thus the precipitation numbers 5 5 5 5 4 3 2 1 0 0 mean complete precipitation in the first four tubes, *i. e.* down to a dilution of 1/80 c.c. of cerebrospinal fluid, partial precipitation of a diminishing order of magnitude in the next four tubes, and absence of precipitation in the ninth and tenth tubes.

THE PRECIPITATION OF NEUTRAL COLLOIDAL GOLD BY NORMAL AND PATHOLOGICAL CEREBROSPINAL FLUIDS.

The general results obtained by us from a large series of cerebrospinal fluids may be stated thus: (1) Normal cerebrospinal fluid never precipitates colloidal gold; (2) paretic cerebrospinal fluid always precipitates colloidal gold, the type of precipitation varying to some degree with the fluid; (3) tabetic cerebrospinal fluid sometimes precipitates colloidal gold.

We have not observed that specificity in the precipitation curves of colloidal gold by tabetic and paretic cerebrospinal fluids which are generally assumed to be characteristic of these diseases. Thus we have often tested specimens of cerebrospinal fluid obtained from definite cases of untreated tabes which gave no precipitation with colloidal gold. On the other hand, cerebrospinal fluids obtained from definite cases of general paresis sometimes gave the precipitation curves which are assumed to be characteristic of tabes. We are inclined to believe that in tabes the cerebrospinal fluid does not precipitate colloidal gold, but when the tabes is associated with paresis, especially in the early cases of tabo-paresis, then the precipitation curve assumed to be characteristic of tabes is obtained. But on this point we do not make any positive statement. A much more extensive series of investigations than we have had the opportunity to perform would be required to settle the question of a definite precipitation curve characteristic of tabes only. It may be remarked that the pathological lesions of the central nervous system are essentially different in the two diseases. In tabes we find a sclerosis of the posterior columns, in general paresis a meningo-encephalitis with an exudate round the vessels of the meninges extending into the brain itself.

The following experimental results illustrate the statement that cerebrospinal fluid obtained from definite cases of general paresis may give precipitation effects which are assumed to be characteristic of general paresis or tabes:

Case.	Precipitation numbers.
1.	5 5 5 5 5 4 4 3 2 1
2.	5 5 5 5 5 4 3 2 1 0
3.	5 5 5 5 4 3 2 1 0 0
4.	4 5 5 3 3 2 1 1 0 0
5.	3 4 5 5 4 4 3 2 1 0
6.	4 5 5 5 5 5 4 3 3 2
7.	3 3 3 5 5 5 5 4 3 2

All these patients showed well-marked signs of general paralysis. It may be observed, however, that only cases (1), (2) and (3) give the precipitation numbers assumed to be typical of general paresis. The precipitation numbers of cases (4) and (5) approximate to those assumed by Miller and Levy to be

characteristic of tabes, whilst the precipitation numbers of cases (6) and (7) suggest cerebrospinal syphilis. In our discussion on the mechanism of the reaction between colloidal gold and cerebrospinal fluid we come to the conclusion that the particular effects observed may be most readily explained by assuming that in pathological cerebrospinal fluid there is an increase in euglobulin, a normal constituent of cerebrospinal fluid, the degree of precipitation of the gold being proportionate to that increase. Since the lesions of tabes are fundamentally different from those of general paresis, it is difficult to appreciate the hypothesis that tabes is characterised by a small increase in the euglobulin of the cerebrospinal fluid, whilst general paresis is accompanied by a large increase in that substance. On the other hand, the presence of a small excess of euglobulin in early cases of general paresis and a large excess in late cases would readily explain the particular types of precipitation curves which have been assumed to be characteristic of tabes and general paresis. On this assumption the cerebrospinal fluid obtained from uncomplicated cases of tabes would not precipitate neutral colloidal gold, whilst cases of tabo-paresis, *i. e.* tabes going on to general paresis, would give the precipitation numbers characteristic of a small increase in the cerebrospinal fluid of euglobulin.

THE PROPERTIES OF THE PRECIPITATING SUBSTANCES.

(a) *Dialysis.*—Contradictory results have been obtained by different observers regarding the capacity of the substances concerned in the precipitation of colloidal gold to pass through parchment or collodion membranes. Cruickshank (1920) definitely found that dialysis in thin collodion membranes did not diminish the capacity of the cerebrospinal fluid to precipitate colloidal gold. On the other hand, Miller, Weston, and Matzkiewitsch (1914) all stated that the precipitating substance is dialysable. In view of the importance of the property in the analysis of the mechanism of the reaction we repeated the experiments.

Paretic cerebrospinal fluid was placed in a small collodion tube and dialysed against ·9 per cent. NaCl. The thickness of the collodion tube was such that equilibrium of the electrolytes occurred within fifteen minutes. After twenty-four hours' dialysis the following results were obtained:

Original cerebrospinal fluid .	5 5 5 5 5 5 4 3 3 0
Dialysed cerebrospinal fluid .	5 5 5 5 5 5 4 3 3 0

The results fully confirm those of Cruickshank.

It is obvious that the precipitating substance shows no tendency whatever to pass through a collodion membrane even after prolonged dialysis, thus disproving the assumption of Matzkiewitsch (1914) that the active substance is a peptone.

(b) *Destruction by high temperature.*—A detailed investigation into the effect of heat on the capacity of a cerebrospinal fluid to precipitate colloidal gold gives interesting results. In fresh cerebrospinal fluid partial destruction of the active agent is produced by heating to 50° C. for five minutes, but this destruction is not complete even after heating to 80° C. for the same length of time. Heating to 100° C. produced complete destruction.

The following figures illustrate this statement:

Original fluid	5 5 5 5 4 3 2 1 0 0
After heating to 50° C. for 5 mins. .	4 4 4 3 3 2 1 0 0 0
„ „ 60° C. „ „ . .	4 4 4 3 2 2 1 0 0 0
„ „ 70° C. „ „ . .	3 3 4 3 2 1 1 0 0 0
„ „ 80° C. „ „ . .	1 1 2 1 1 0 0 0 0 0
„ „ 100° C. „ „ . .	0 0 0 0 0 0 0 0 0 0

There is no indication from these figures of a definite temperature of destruction by heat such as is usually assigned to the heat of coagulation of a protein.

Another fact which often appears in this kind of experiment is the tendency of the precipitation curve of a heated fluid to take on a tabetic type. This is illustrated by the following results:

Original fluid	3 3 4 4 4 4 3 3 2 1
Heated to 80° C. for 5 mins. . .	1 2 3 5 5 4 4 3 2 0
„ 90° C. „ „ . . .	0 0 2 3 3 2 1 0 0 0
„ 100° C. „ „ . . .	0 0 0 0 0 0 0 0 0 0

The divergent results obtained by different observers on the effect of heat on cerebrospinal fluid are probably due to the time which has elapsed between the withdrawal of the fluid from the body and the heating experiments. Although the precipitating substance is very stable and persists in cerebrospinal fluids for a considerable period of time (months), yet old cerebrospinal fluid has its precipitating property destroyed by heating to 60° C. for 5 minutes. This result is probably due to the increased alkalinity of old cerebrospinal fluid owing to the evolution of CO_2 and the consequent formation of Na_2CO_3 from $NaHCO_3$ in the fluid. The following figures show the influence of reaction on the destruction of the precipitating substance by heat. The original unheated fluid gave the precipitation numbers 5 5 5 5 5 4 3 2 1 0. Heated to 80° C. for five minutes:

Original fluid . .	Reaction	pH 7·6 .	0 0 0 0 0 0 0 0 0 0
„ „ plus acid	„	pH 7·1 .	1 1 1 1 1 0 0 0 0 0
„ „ „ „	„	pH 6·5 .	2 5 5 5 2 0 0 0 0 0

In a definitely alkaline medium complete destruction occurred at 80° C.; in a neutral medium a small quantity of the active substance remained undestroyed; and in an acidic medium a quantity of active substance capable of giving a definite tabetic precipitation curve was left. It may be observed that the quantities of acid used had no effect on the precipitation of the colloidal gold.

(c) *Precipitation by ammonium sulphate.*—We find, in agreement with other observers (Weston, Cruickshank), that the active agent contained in paretic cerebrospinal fluid concerned in the precipitation of colloidal gold is completely precipitated by half saturation with ammonium sulphate. Thus the following reactions were given by (*a*) paretic cerebrospinal fluid, and (*b*) the solution obtained by precipitating 2 c.c. of the same cerebrospinal fluid with half saturated ammonium sulphate, and redissolving the precipitate in 2 c.c. of water:

Original fluid	5 5 5 5 4 3 2 1 0 0
Solution of Am_2SO_4 ppt. . . .	5 5 5 5 5 5 4 3 3 2

The augmented effect which is invariably given by the ammonium sulphate precipitate suggests that a substance inhibiting the precipitation of colloidal

gold is contained in cerebrospinal fluid, and that this inhibitory substance is not completely precipitated by half saturation with ammonium sulphate.

(d) *Precipitation by alcohol.*—It has been shown by Mellanby (1907) that alcohol at 15° C. not only precipitates protein from serum but renders it insoluble. If, however, the alcohol and the serum be first cooled to 0° C. before mixing, then the protein so precipitated is readily soluble in water. On adding alcohol, cooled to − 10° C., to the extent of 50 per cent. to a paretic cerebrospinal fluid, we found that the active agent was completely precipitated, and when redissolved in water gave a solution possessing an activity slightly greater than that of the original fluid:

Paretic cerebrospinal fluid . . .	5 5 5 5 5 4 3 2 1 0
50 per cent. alcohol ppt. in water . .	5 5 5 5 5 5 5 5 4 4 0

Apparently, after alcohol precipitation also, some inhibitory substance remains in the filtrate in a manner comparable to that observed with ammonium sulphate precipitation.

The hypothesis that a substance inhibiting the precipitation of colloidal gold is present in cerebrospinal fluid, and that this substance is not readily precipitated by ammonium sulphate or alcohol, was investigated more closely in the case of precipitation by alcohol.

4 c.c. of alcohol, cooled to − 5° C., were added to 2 c.c. of cerebrospinal fluid. The precipitate was obtained by spinning the mixture in a centrifuge. This precipitate was dissolved in 2 c.c. of 0·9 per cent. NaCl. The following give the precipitation numbers of (A) the original cerebrospinal fluid, (B) the precipitate dissolved in 0·9 per cent. NaCl, (C) the filtrate freed from alcohol and made up to the original volume of cerebrospinal fluid, and (D) the fluid produced by mixing quantities of (B) and (C) equal to those contained in the original cerebrospinal fluid:

(A) Cerebrospinal fluid	3 4 5 4 3 2 1 0 0 0
(B) Alcohol precipitate	5 5 5 4 3 2 1 0 0 0
(C) Filtrate freed from alcohol . .	0 0 0 0 0 0 0 0 0 0
(D) Mixtures of (B) and (C) in equivalent proportions	3 4 4 4 3 2 1 0 0 0

The results show definitely (1) that the active agent is entirely precipitated unimpaired by cold alcohol, and (2) that some inhibitory substance contained in cerebrospinal fluid is not precipitated by alcohol. The results (A) and (B) show how readily a so-called tabetic precipitation curve becomes a paretic curve.

FACTORS WHICH DETERMINE THE SENSITIVITY OF COLLOIDAL GOLD TO PRECIPITATION BY CEREBROSPINAL FLUIDS.

We have never observed the precipitation of *neutral* colloidal gold by cerebrospinal fluid obtained from a normal individual. The sensitivity of a gold sol, however, is profoundly influenced by its reaction. Therefore a normal cerebrospinal fluid may give a tabetic or paretic precipitation curve with a sensitised gold solution. Similarly, a paretic fluid may give no precipitation with a desensitised gold sol and so similate a normal fluid. Generally stated, neutral salts have very little influence on colloidal gold, but the tendency to precipitate with cerebrospinal fluid is greatly augmented by traces of acid and depressed by traces of alkali.

(1) *Potassium oxalate.*—Varying amounts of potassium oxalate have very little influence on the precipitation of colloidal gold by cerebrospinal fluid, provided that the amount of oxalate used in the preparation of the colloidal gold is less than that required to produce a turbid preparation. Three gold sols were prepared in the way previously described, except that in the first case 0·8 c.c. of potassium oxalate (1 per cent.) had been added to each 100 c.c. of distilled water, in the second case 1·0 c.c. of oxalate, and in the third case 1·6 c.c. of oxalate. These three gold sols were perfectly clear and of a characteristic red colour. The precipitation numbers with the same paretic cerebrospinal fluid were:

Neutral gold containing 0·8 c.c. pot. oxalate (1%) per 100 c.c	5	5	5	5	4	3	2	1	0	0
„ „ 1·0 c.c. „ „ „	5	5	4	4	3	2	2	1	0	0
„ „ 1·6 c.c. „ „ „	5	5	4	4	3	3	2	1	0	0

Double the amount of potassium oxalate contained in the gold sol diminished to a very small degree only its precipitation by paretic cerebrospinal fluid.

(2) *Acid.*—Acidic colloidal gold in contra-distinction to neutral gold is readily precipitated by *normal* cerebrospinal fluid, the degree and type of precipitation varying with the reaction of the gold. The experimental results given illustrate this statement.

Neutral colloidal gold was acidified to varying degrees by the addition to it of normal oxalic acid. *Normal* cerebrospinal fluid gave the following precipitation numbers of these acidified gold sols:

Neutral gold sol	0	0	0	0	0	0	0	0	0	(A)
Sol containing N/5000 acid	0	0	4	3	2	1	1	0	0	(B)
„ „ N/3000 „	2	4	5	5	4	2	1	0	0	(C)
„ „ N/2500 „	5	5	5	5	3	2	1	0	0	(D)

According to the classification of Miller and Levy the absence of precipitation (A) is characteristic of normal cerebrospinal fluid, (B) simulates early tabes; (C) advanced tabes and (D) general paresis. In fact, if the test had been made with a cerebrospinal fluid of unknown origin, results indicating a normal, tabetic or paretic individual would be obtained if the reaction of the gold sol varied so little as N/2500 acid. The value of the colloidal gold test, therefore is absolutely dependent upon the neutrality of the gold sol used. This fact emphasises the necessity of using a method for the preparation of a gold sol such as we have described, *i. e.* by the use of reagents neutral in reaction. A second fact is evidenced from the above results—normal cerebrospinal fluid contains all those substances which are responsible for the peculiar precipitation effects which are assumed to be pathognomonic of neuro-syphilis.

(3) *Alkali.*—Just as acid increases the sensitivity of colloidal gold towards precipitation by cerebrospinal fluid, so alkali diminishes this tendency. The degree of diminution with alkali is of the same order as the increase observed with acid, except that only minute quantities of alkali may be added without producing spontaneous precipitation of the gold sol. The following figures give the precipitation numbers of colloidal gold to which varying small quantities of alkali had been added by a paretic cerebrospinal fluid:

Reaction of gold.											
Neutral	5	5	5	5	5	4	3	2	2	0
N/50,000 alkali	4	5	5	5	4	4	3	2	2	0
N/25,000 „	4	4	4	4	3	2	2	1	0	0
N/10,000 „ . : . .	4	3	2	2	1	1	1	1	0	0

It may be observed that the quantities of alkali are only one-tenth of the quantities of acid used in a corresponding experiment. From these figures we see that minute quantities of alkali convert a paretic into a tabetic precipitation curve, and larger quantities a paretic into practically a normal curve. In fact, just as a gold sol sensitised with acid can give a paretic precipitation curve with a normal cerebrospinal fluid, so a gold sol desensitised with alkali can show no precipitation with a paretic cerebrospinal fluid. The results again emphasise the necessity of working with a neutral gold sol.

(4) *Age.*—Although it is absolutely essential to use freshly distilled water to prepare colloidal gold, yet, when the gold sol has been prepared, the time which has elapsed between its preparation and the testing of a fluid on it is of minor importance. It is necessary to keep the gold sol in a stoppered bottle of hard glass, otherwise spontaneous precipitation occurs. A gold sol left in contact with the air becomes more sensitive to precipitation, probably owing to the solution in it of acids (CO_2, etc.) from the atmosphere. This statement is illustrated by the following results:

Fresh gold sol with normal C.S.F. . . .	0	0	0	0	0	0	0	0	0	0
Same gold sol 24 hours later with same C.S.F. .	0	1	3	4	3	2	1	0	0	0

During the 24 hours in which the gold sol was left open to the air the atmosphere was humid and foggy. Possibly as a routine practice it is better to use freshly prepared colloidal gold.

THE PRECIPITATION OF COLLOIDAL IRON BY CEREBROSPINAL FLUID.

An analysis of the precipitation numbers of colloidal gold by luetic cerebrospinal fluids indicates that in these reactions not only a precipitating agent but also an anti-precipitating agent is involved. On the accepted hypothesis of colloidal solutions the gold sol is precipitated by a positively charged colloid contained in the cerebrospinal fluid. It appeared possible that the substances inhibiting precipitation might be the inorganic salts contained in the cerebrospinal fluids. Quantitative experiments, however, showed that electrolytes, even in large amounts, show little tendency to inhibit the precipitation of antagonistic colloids. They tend rather to bring one colloid out of solution and so render the second colloid more unstable. It appeared probable, therefore, that the inhibitory substance was a second colloid. This hypothesis was confirmed by the observation that cerebrospinal fluids precipitate positively charged colloidal iron in definite dilutions. The following experimental result illustrates this statement:

Colloidal iron (0·05 per cent.) was substituted for colloidal gold in a test made in the usual way on paretic cerebrospinal fluid. The precipitation numbers for the colloidal iron were 5 5 5 0 0 0 0 0 0 0. The precipitation numbers for the same cerebrospinal fluid in colloidal gold were 5 5 5 5 5 5 4 3 3 0. It may be assumed, therefore, that this cerebrospinal fluid contained not only positively charged colloid in relatively large quantities, which pre-

cipitated colloidal gold, but also a negatively charged colloid in smaller concentrations which precipitated colloidal iron. It may be asked why these two oppositely charged colloids do not produce mutual precipitation in cerebrospinal fluid. The absence of precipitation is probably due to the colloid which is present in excess keeping the other colloid in solution just as comparatively large quantities of colloidal iron keep colloidal gold in solution.

THE MUTUAL PRECIPITATION OF ANTAGONISTIC COLLOIDS.

The factors involved in the precipitation of colloidal gold by luetic cerebrospinal fluids may be analysed by determining (1) the precipitation of colloidal gold by colloidal iron, and (2) the precipitation of colloidal gold by albumen, globulin and serum.

(1) *The Mutual Precipitation of Colloidal Gold and Colloidal Iron.*

Colloidal gold carries a negative charge, colloidal iron carries a positive charge. Therefore in suitable dilutions these two colloidal solutions mutually precipitate one another. The quantitative relations involved in these precipitations were determined by precipitating colloidal gold by colloidal iron in a manner similar to that used in the cerebrospinal fluid tests, *i. e.* colloidal iron 0·5 per cent. was substituted for the cerebrospinal fluid. The quantity of colloidal gold used was constant (5 c.c.); the quantities of colloidal iron and the degrees of precipitation are shown:

Colloidal gold (0·01 per cent.) in each tube, 5 c.c.:

Colloidal iron (0·5 per cent.) .	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$
Degree of ppt. . . .	0	0	0	0	5	5	5	5	5	0

From these figures it may be seen that approximately 0·00005 grm. of colloidal iron completely precipitate, 0·0005 grm. of colloidal gold, and that this precipitation continues until the iron is reduced to 0·000002 grm. Quantities of colloidal iron greater than 0·0005 grm. keep the gold in solution and 0·0005 grm. of colloidal gold keep quantities of iron less than ·000002 grm. in solution. In fact, on adding continually diminishing quantities of colloidal iron to a constant quantity of colloidal gold three zones may be observed: (a) the first zone in which the iron keeps the gold in solution, (b) the second zone in which all the iron and all the gold is precipitated, the colour of the precipitate varying from brown when the iron is relatively large in quantity to purple when the gold predominates, and (c) a zone in which the gold keeps minute quantities of iron in solution. There is no definite chemical relation between the amount of iron and gold precipitated. It is obvious, however, that the effects observed in the precipitation of neutral colloidal gold by neutral colloidal iron are not comparable to the precipitation phenomena obtained when colloidal gold is precipitated by a luetic cerebrospinal fluid. In fact we cannot assume that the sole agent involved in the precipitation of colloidal gold by luetic cerebrospinal fluids is a positively charged colloid.

(2) *The Precipitation of Colloidal Gold by Emulsoid Colloids.*

Colloidal gold is not precipitated by egg-albumen, gelatine, starch or glycogen in any dilution. Presumably therefore these emulsoid colloids carry

a negative charge similar to that carried by colloidal gold. Precipitation, however, is produced by very small quantities of "eu" globulin when dissolved in 0·9 per cent. NaCl, and to a less degree by this protein when present in serum.

(a) *Euglobulin.*—The precipitation of colloidal gold by euglobulin is broadly similar to the precipitation of gold by iron. Small quantities of globulin give the typical paretic precipitation curve; larger quantities tend to keep the gold in solution.

Serum globulin was precipitated by diluting serum with ten volumes of distilled water and bringing the mixture to the iso-electric point for globulin by the cautious addition of acetic acid. The precipitated globulin was obtained as a compact mass by spinning the fluid, and, after pouring off the supernatant liquid, was dissolved in a volume of ·9 per cent. NaCl equal to that of the original serum. It may be observed that the globulin so obtained is "eu" globulin only, and not a mixture of "eu" and "pseudo" globulin, which is obtained on half saturating serum with ammonium sulphate. This globulin solution diluted to one part in a thousand parts of salt solution gave the following precipitation numbers:

0·1 per cent. of globulin solution . . 5 5 5 4 3 2 1 0 0 0

The actual strength of globulin solution used was 0·0005 per cent., so that, from the above figures, 0·0000001 grm. of globulin precipitates completely 5 c.c. of colloidal gold. It is interesting to observe that a minimal quantity of 0·000002 grm. of colloidal iron also completely precipitates 5 c.c. of colloidal gold—an amount which is twenty times as great as the globulin. More concentrated solutions of globulin gave precipitation numbers which indicate that euglobulin in relatively large quantities tends to hold gold in solution in a manner comparable to that observed with colloidal iron. Thus 1 per cent. of globulin solution gave the precipitation numbers 3 3 3 5 5 5 4 0 0 0. The capacity of globulin to keep gold in solution is not equal to that shown by colloidal iron. We discuss this question more fully when we consider the general questions involved in the precipitation curves of luetic cerebrospinal fluids.

(b) *Serum.*—The precipitation of colloidal gold by serum appears to be due entirely to the euglobulin contained within it. But it is evident that some substance inhibiting the precipitation of colloidal gold by euglobulin is also present in serum, as shown by the following precipitation figures for serum:

(A) Serum 0·1 per cent. 4 4 3 3 0 0 0 0 0 0
(B) Euglobulin from a comparable volume of serum 5 5 5 4 3 2 0 0 0 0
(C) "Eu" and "pseudo" globulin from a comparable volume of serum . . 4 4 3 3 0 0 0 0 0 0

From (A) and (B) it may be seen that euglobulin prepared from serum is a more effective precipitant than the serum itself—that is, that serum contains some substance which inhibits the precipitation of colloidal gold by euglobulin. The result (C) shows that the precipitate obtained from serum by half saturation with ammonium sulphate, when redissolved in water, has the same precipitation effect as the original serum. Therefore this precipitate contains not only the precipitating agent but also the anti-precipitant. Broadly speaking, the two proteins precipitated from serum by half saturation with ammonium sulphate are "eu" and "pseudo" globulin. Euglobulin in minute

quantities precipitates colloidal gold. Therefore we must assume that pseudoglobulin, or some substance associated with the pseudoglobulin in its precipitations, is the agent contained in serum which inhibits precipitation.

DISCUSSION OF RESULTS.

(1) The facts observed in the precipitation of colloidal gold by cerebrospinal fluid may be briefly summarised:

(*a*) Neutral colloidal gold is not precipitated by normal cerebrospinal fluid, but gives typical precipitation curves with luetic cerebrospinal fluid.

(*b*) Colloidal gold which has been sensitised by the addition of acid gives a tabetic or paretic precipitation curve with normal cerebrospinal fluid according as the quantity of acid added, and therefore the degree of sensitisation is small or large. Similarly, colloidal gold which has been desensitised by the addition of alkali gives a tabetic precipitation curve with paretic cerebrospinal fluid. From this it follows that the precipitating agents concerned in the luetic reactions are contained in a subminimal quantity in normal cerebrospinal fluid and in increasing quantities in tabetic and paretic cerebrospinal fluids.

(*c*) The precipitating agents are nondialysable, precipitated unchanged by half saturation with ammonium sulphate or by cold alcohol, and are slowly destroyed by heat at temperatures depending upon the reaction of the cerebrospinal fluid. These facts indicate that the precipitating factors are either "eu" or "pseudo" globulin, or a mixture of them.

(2) The precipitation of colloidal gold by oppositely charged colloids such as colloidal iron or euglobulin may be summarised:

(*a*) Colloidal iron or euglobulin totally precipitates gold over a large range of concentrations, but the precipitation is mutual and complete over this range.

(*b*) Large quantities of iron or globulin keep the gold in solution, and, similarly, colloidal gold keeps minute quantities of colloidal iron or globulin in solution. In fact, a colloid is not precipitated by an oppositely charged colloid if present in relatively large quantities.

A comparison of the results summarised in (1) and (2) appears to indicate that the phenomena observed in the precipitation of colloidal gold by luetic cerebrospinal fluid are completely analogous to the mutual precipitations of antagonistic colloids. But the similarity is only superficial, for whereas large quantities of colloidal iron keep colloidal gold in solution, the typical tabetic curve is obtained intermediate between the absence of precipitation by normal cerebrospinal fluid and full precipitation by paretic cerebrospinal fluid. In other words, normal cerebrospinal fluid contains only a trace of the precipitating agent and produces no observable effect on neutral colloidal gold, tabetic fluid contains a larger quantity of this substance and gives its characteristic curve with precipitation in the middle tubes, whilst paretic fluid contains the greatest quantity and gives complete precipitations at high concentrations.

The presence of antagonistic colloids in cerebrospinal fluid is evident, since this fluid precipitation not only negatively charged colloidal gold, but also positively charged colloidal iron. It is probable, therefore, that the phenomena observed in the precipitation of colloidal gold by luetic fluids may be explained on the assumption of a balanced reaction between these oppositely charged

colloids. This assumption is supported by the observations made on the precipitation of colloidal gold by (*a*) euglobulin and (*b*) a mixture of "eu" and "pseudo" globulin. The results so obtained may be appreciated if it is assumed that euglobulin actively precipitates colloidal gold, whilst pseudo-globulin tends to keep gold in solution. From a consideration of these facts we put forward the following hypothesis: All cerebrospinal fluids contain "eu" and "pseudo" globulin, the quantities of these substances increasing as we pass from normal to tabetic and from tabetic to paretic cerebrospinal fluid. "Eu" globulin is the active agent which precipitates colloidal gold; "pseudo" globulin tends to keep the gold in solution and antagonises the precipitating action of "eu" globulin. "Eu" globulin, being soluble only in electrolytes, tends to come out of solution on dilution, and therefore its precipitating action is augmented on dilution; "pseudo" globulin, on the other hand, is soluble in water, and therefore its capacity to hold gold in solution is diminished by dilution in a magnitude equal to the degree of dilution. The increased precipitating action of euglobulin on dilution appears to be responsible for the precipitation curve which has been described as typical of tabes. Probably all cerebrospinal fluids contain a constant quantity of "pseudo" globulin, but variable quantities of "eu" globulin. The tabetic curve is due to a small increase in "eu" globulin, which manifests its action only in the intermediate dilutions when its tendency to come out of solution is augmented to a maximal extent by dilution. In general paresis, on the other hand, the "eu" globulin is increased to such an extent that it overcomes the inhibitory action of the "pseudo" globulin and precipitates colloidal gold in the first dilutions of the gold sol test.

It is possible, however, that "pseudo" globulin as well as "eu" globulin may be increased in certain luetic conditions. Thus in cerebral syphilis the cerebrospinal fluid has been stated to give the following precipitation numbers —0 0 0 5 5 5 4 3 2 1. On our hypothesis the absence of precipitation in the first tubes would be due to an increased quantity of "pseudo" globulin preventing the precipitation of the gold by the "eu" globulin, but on increasing dilution the "eu" globulin tends to come out of solution, and this increased tendency to precipitation overcomes the inhibitory action of the "pseudo" globulin and precipitations in high dilutions result. This explanation is more probable than the assumption that cerebral syphilis is associated with a large increase of "eu" globulin in the cerebrospinal fluid. "Eu" globulin has to be present in massive quantities before it can keep gold in solution.

Finally, we propose to discuss at a later date the question whether the "eu" globulin found in syphilitic cerebrospinal fluids, although similar in physical properties to the "eu" globulin of serum, yet differs from it in chemical composition, and is a specific result of the action of the syphilitic virus.

SUMMARY.

(1) Colloidal gold, neutral in reaction, may be prepared by adding 1 c.c. of $AuCl_3$ (1 per cent.) to 100 c.c. of boiling potassium oxalate (0·01 per cent.).

(2) Precipitation of neutral colloidal gold is always produced by paretic cerebrospinal fluid, sometimes by tabetic fluid, but never by normal fluid.

Cerebrospinal fluid obtained from definite cases of general paresis often gives the precipitation curve assumed to be characteristic of tabes.

(3) The precipitating substance is (*a*) non-dialysable, (*b*) precipitated by half saturation with ammonium sulphate, (*c*) precipitated by 50 per cent. alcohol at 0° C., (*d*) destroyed by heat at temperatures depending upon the reaction of the fluid and the duration of heating.

(4) Neutral colloidal gold, sensitised by the addition of acid, may give a tabetic or paretic precipitation curve with normal cerebrospinal fluid depending on the degree of sensitisation, and *vice versâ* on desensitisation by alkali. These facts emphasise the necessity of working with a neutral colloidal gold.

(5) Neutral salts have a small influence only on the sensitivity of colloidal gold to precipitation by cerebrospinal fluid.

(6) All cerebrospinal fluids contain not only a positively charged colloid which precipitates colloidal gold, but also a negatively charged colloid which precipitates colloidal iron, and therefore antagonises the precipitating action of the former on a gold sol. The positive colloid appears to be a "eu" globulin, the negative colloid a "pseudo" globulin. In normal cerebrospinal fluids the "eu" and "pseudo" globulins are present in small quantities only, and the precipitating action of the "eu" globulin is balanced by the inhibitory action of the "pseudo" globulin unless a gold sol is used which has been sensitised by acid (*i.e.* a sol in which the tendency of the gold to precipitate has been augmented). In cerebrospinal fluids which give the tabes precipitation curve the "eu" globulin is increased in amount to a moderate extent, and to a larger extent in fluids which give the paretic precipitation curve. Possibly the precipitation curves stated to be characteristic of cerebrospinal syphilis, *i.e.* absence of precipitation in low dilutions and complete precipitation in high dilutions, are due to a large increase of both "pseudo" and "eu" globulins in the cerebrospinal fluid.

We desire to place on record our thanks to Dr. le Marquand, of the Claybury Mental Hospital, Dr. S. R. Tattersall, of the Lancaster Mental Hospital, and Dr. Stebbings, of Lambeth Infirmary, for many specimens of cerebrospinal fluid.

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SOME NEW PRINCIPLES IN BACTERIAL IMMUNITY THEIR EXPERIMENTAL FOUNDATION, AND THEIR APPLICATION TO THE TREATMENT OF REFRACTORY INFECTIONS.

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Received for publication May 24th, 1923.

GENERAL STATEMENT OF THEORIES AND RESULTS.

In spite of a great accumulation of experimental and clinical data concerning the principles and the practical application of bacteriotherapy, serotherapy, and prophylaxis, the therapeutic results obtained, magnificent though they may often be, are strangely lacking in uniformity.

The effects, for example, of specific vaccines upon acute or chronic infections may be in one case almost uniformly favourable, and, in another, completely negative. The same statements apply also to the prophylactic and therapeutic bacterial anti-sera.

Now, when one reviews this field of experience, and searches for possible reasons for the lack of uniform results, one is struck by the fact that, in general, the successes of bacterial therapy and prophylaxis are obtained with micro-organisms of the non-acid-fast and Gram-negative varieties. Failure, on the contrary, is the general rule where acid-fast and Gram-positive bacteria are in question. It must be understood that we are here considering only the bacterial bodies themselves, and not their soluble products, such as toxins. It is true that we cannot claim for this general principle the validity of a clear-cut and universal law, for there are certain cases in which the rule does not altogether hold good: for example, partial successes have been obtained with pneumococcal antiserum, and with staphylococcal vaccines; and, on the contrary, failure is very far from rare with vaccines of Gram-negative bacteria such as the gonococcus.

Nevertheless the rule holds good in a sufficiently large proportion of cases to give us an important clue towards the discovery of the underlying truth.

Since acid-fastness and Gram-positiveness have been clearly proved to be associated with the presence in the bacterium of certain waxy, fatty or lipoid substances,* it seemed probable, *à priori*, that these substances were the cause of failure in immunisation. Owing to their power of protecting the specific bacterial proteins and preventing their liberation from the body of the bacterium, these substances could be conceived as preventing or retarding the production of the sole adequate stimulus for the immunity reaction of the infected organism.

* In the remainder of this paper the waxy, fatty or lipoid substances of bacteria of all varieties will be referred to as "lipoidal" substances.

Support for this view is to be found in the work of Douglas (1921) on the tryptic digestion of acetone-extracted bacteria. He had invariable success in digesting and obtaining immunity responses with Gram-negative bacteria, such as the dysentery bacillus, the typhoid bacillus and the gonococcus, whereas the Gram-positive organisms either failed altogether to digest (staphylococcus) or underwent apparent digestion, but gave rise to no immune bodies (*B. welchii*).

In view of these circumstances it seemed desirable to attack the problem by attempting the removal, by extraction, of the "lipoidal" fraction of some representative acid-fast and Gram-negative bacteria. The tubercle bacillus was first selected, as having a highly-developed lipoid constituent, and as being, in many respects, a very robust and resistant organism.

A number of processes involving simple extraction with various fat-solvents were unsuccessfully tried. For instance, a large quantity of *B. tuberculosis* was killed with heat, dried, ground up and extracted with acetone in a Soxhlet apparatus for 34 days without intermission at 65° C. At the end of this time, although large quantities of "lipoidal" substances had been extracted, the bacilli were found to be still quite acid-fast.

It thus became clear that something more than simple extraction with ordinary fat-solvents was necessary if the removal of the acid-fast substances was to be effected.

Fortunately a clue to the problem was forthcoming in the shape of the following fact, which had been observed by various workers, including myself, viz. that a tuberculous tissue fixed with formalin often tends to show far fewer tubercle bacilli, when suitably stained, than the same tissue fixed with perchloride of mercury.

Might it not be, then, that formalin removes the acid-fast substances, and thus prevents the demonstration of the bacilli by staining?

To test this hypothesis, tubercle bacilli, grown on the usual media, were treated with strong formalin for various lengths of time, but the result appeared negative, for the bacilli remained acid-fast, although their appearance underwent a definite change in the direction of increased granularity.

Evidently, then, it could not be the formalin alone that caused the loss of acid-fastness. In the preparation of tissues for section, however, a number of other reagents are employed, including fat-solvents such as alcohol, xylol or chloroform. It therefore seemed to me probable that the phenomenon might be due to the successive application of formalin and fat-solvents to the bacilli.

Experiments at once showed that, with a suitable technique, the tubercle bacillus could readily be rendered non-acid-fast in this manner.

Now, in view of our knowledge that bacteria killed with formalin retain their antigenic power, as they do also after simple extraction with acetone (Douglas), there was good reason to hope that the residue of the tubercle bacillus, after treatment with formalin and extraction with acetone, would retain all or part of the specific properties of the bacillus, and that this residue could be used for immunisation. Numerous experiments demonstrated the correctness of this reasoning, as will be seen later.

The facts so far established led me to the expectation that it would be possible to deal with other acid-fast bacteria in the same manner, and also, by

a similar process, to reduce Gram-positive micro-organisms to the Gram-negative condition. This was found to be the case, and it was further shown that in this state they no longer resisted the digestive action of trypsin.

The next step achieved was the demonstration of the capacity of the "defatted" bacteria to react with specific antisera, and this led on to the experimental proof of their antigenic action in the animal body, where they gave rise, on injection, to the production of the well-known "immune substances," such as bacteriolysins, complement-fixing bodies, precipitins and agglutinins.

Finally, a series of experiments was carried out by which it was established that these antigens can exert definite curative effects on animals suffering from bacterial infection.

In order to complete this section of the work, it was thought desirable to discover whether this somewhat heroic process of formalin-acetone extraction exercised a destructive effect on the essential antigen of Gram-negative bacteria, which might possibly prove to be more fragile than their Gram-positive brethren.

On subjecting *B. typhosus* to the process, it was shown that the extracted bacilli had retained their ability to cause the production of immune substances, such as specific precipitins and complement-fixing substances.

Neither in this case nor in any of the foregoing cases has it been established that none of the antigenic power is lost, but it is clearly proved that any loss that may occur can only be partial and therefore unimportant.

As a corollary of the main lines of research just described, a second and closely related train of thought suggested itself to me.

Pasteur's classical demonstration of the immunising properties of anthrax cultures "attenuated" by growth at 42°–43° C. has hitherto received nothing but the vaguest of explanations. Starting now from the new knowledge that "defatted" anthrax bacilli can be digested by trypsin, whereas the untreated bacilli are in the main indigestible, it seemed a reasonable hypothesis that growth at 42°–43° C. gives rise to a deficient formation of the normal "fatty" constituent, and thus renders the bacilli more easily susceptible to the digestive action of the body-fluids. Such bacilli, when injected into the living body, would be, at least in part, readily disintegrated, and the release of their antigen would cause a rapid immunity-response in the animal, before the Gram-positive, and therefore more resistant, bacilli developed in sufficient number to overwhelm the normal resistance of the body. In view of the foregoing considerations one was able to predict that growth at 42°–43° C. would be found to give rise to a weakening or disappearance of the Gram-positiveness of anthrax-bacilli. A few simple experiments proved this to be the case not only for that bacillus, but also for a stock laboratory strain of *Staphylococcus aureus*.

This latter organism was also shown to be digestible by trypsin after the formalin-acetone treatment, whereas Douglas had found it indigestible after acetone extraction alone.

The formalin-acetone process, whereby a relatively complete separation of "fatty" substances from the specific proteins is attained, has enabled me to throw light upon another set of unexplained phenomena.

It is well known that the subcutaneous injection of dead tubercle bacilli or staphylococci causes a severe local reaction with infiltration and sometimes necrosis, often apparently unaccompanied by any noteworthy general disturbance. In the light of our new knowledge the probability suggested itself that the local disturbance might be due entirely to the highly indigestible and irritating "fatty" constituents of the bacilli, and in no way to their specific antigenic proteins. This hypothesis was readily tested by the subcutaneous injection into suitable animals of the acetone extracts of tubercle bacilli and of staphylococci, previously treated with formalin, and the theory was shown to accord completely with the facts. The injection into normal animals of tubercle and staphylococcal "lipoids" gave rise to infiltrations which tended to persist for a long time; and in one case the undissolved mass of "lipoids" ulcerated through the skin and discharged itself, while a larger dose of defatted bacilli than corresponded to the dose of "lipoids" caused no local reaction in the same animal. We may take this as an additional proof that the "lipoids" of the bactericidal body form an important obstacle to the solvent and protective action of the body-fluids.

THE PROCEDURE FOR OBTAINING "DEFATTED" BACTERIAL ANTIGENS.

Definition of the Term "Defatted."

The term "defatted" is not intended to indicate that all the "lipoidal" substances present in the micro-organism have been removed. It merely implies that certain "lipoidal" substances, soluble in acetone after formalin-treatment, have been taken away.

In the case of the acid-fast and Gram-positive bacteria, the indication that the extraction has proceeded far enough for our purpose is the change of the staining reactions to non-acid-fastness or Gram-negativeness respectively.

Preparation of B. tuberculosis *Antigen.*

Tubercle bacilli are grown on the surface of glycerin-broth or other suitable liquid medium for a sufficient length of time, *e.g.* 2–3 weeks.

The fluid is decanted off and the mass of tubercle bacilli is transferred to an agate mortar. Some pure formalin can be added to the flask after the liquid has been removed in order to render the manipulations safer.

To the bacilli in the mortar a few drops of formalin (40 per cent. formaldehyde) are added, and the whole is ground up to a thick homogeneous paste. More formalin is then gradually added, with constant grinding, until 150–200 c.c. of formalin have been added to each 5 grm. of bacilli weighed wet.

The suspension of tubercle bacilli in formalin is now transferred to a flask fitted with a reflux condenser, and the flask is deeply immersed in a water-bath, which is heated to 100° C. and maintained at that temperature for 4 hours.

The suspension is now filtered through calcium-free filter-paper and the residue is washed three or four times with acetone until about 100° c.c. of acetone has been used.

The residue is next transferred to an extraction-thimble and this is placed

in a Soxhlet apparatus with *ground-glass joints.* The whole is put in a water-bath in such a way that the extractor is partly immersed and the top of the thimble is approximately at the same level as the water in the bath. By this means the acetone around the thimble is kept at the boiling-point. The water-bath is kept at a temperature of 65°–70° C. and the extraction is allowed to proceed for 20–24 hours.

At the end of this time the insoluble residue is taken out, dried and ground in a sterile mortar. If the bacilli are not then entirely non-acid-fast the process is repeated, *i. e.* the dried bacilli are again ground in formalin, heated for 4 hours at 100° C., washed with acetone, extracted with acetone for 20–24 hours at 65°–70° C., dried and ground up. It may be necessary to repeat the process a third, or even a fourth time, if the organisms are very resistant to extraction.

Preparation of suspension of T.B. antigen.—0·1 grm. of the dried extracted tubercle bacilli is weighed out with aseptic precautions and ground up very thoroughly in a sterile agate mortar.

Sterile saline solution (0·9 per cent.) is added drop by drop until, with continued grinding, a thick homogeneous paste is obtained. This is diluted gradually to 10·0 c.c. with the saline while grinding. It is then put in a sterile centrifuge-tube, which is closed with a sterile rubber stopper and centrifuged for 5–8 minutes at a speed of about 3000 revolutions per minute, until the milky supernatant fluid shows no granulation when examined with a six-magnification lens.

The supernatant fluid, when pipetted off and suitably diluted (see below) with sterile 0·9 per cent. saline containing 0·15 per cent. formalin, constitutes the antigen-suspension. The residue, together with a certain quantity of fluid at the bottom of the centrifuge-tube, must now be dried and weighed in order to estimate, by subtraction, how much of the original 0·1 grm. of "defatted" bacilli is contained in the antigen-suspension. Moreover, a slight correction is necessary in the calculation, for by drying the centrifuged deposit with a certain quantity of saline the weight of the salt in the saline has been added to that of the residual "defatted" bacilli.

The quantity of fluid left in the centrifuge tube is clearly equal to 10 c.c. minus the number of c.c. pipetted off (which must be measured), and the weight of NaCl in grammes is, of course, 0·9 per cent. of the quantity of fluid left in the tube.

Preparation of stock suspension.—Having thus ascertained the actual weight of "defatted" organisms in the suspension, the latter is diluted with sterile 0·9 per cent. saline, containing 0·15 per cent. formalin, in such a manner that *one cubic centimetre* of the fluid suspension contains *one fifth of a milligramme of dry "defatted" tubercle bacilli.*

Preparation of suspension for use.—These are made up from the stock suspension as required, and always contain a definite weight of "defatted" T.B. per volume of fluid. The quantities vary according to the purpose for which they are to be used—as, for instance, for treatment of infections; for injection into normal animals in the production of bacteriolysins, precipitins, complement-fixing substances and agglutinins, or for use as test-suspensions in complement-fixation or agglutination-experiments.

Preparation of "Defatted" Antigens of Bacteria other than B. tuberculosis.

By the process just described, "defatted" antigens of human and bovine tubercle bacilli, *B. anthracis*, staphylococcus, streptococcus, pneumococcus, *B. diphtheriæ*, *B. lepræ*, *B. typhosus*, gonococcus, etc., have been prepared. The method is doubtless applicable to all bacteria, whether acid-fast, Gram-positive or Gram-negative.

The ease and rapidity of extraction differ in different bacteria. For example, some strains of the streptococcus have been found very refractory. Again, a bacterium grown under varying conditions, as regards culture-medium and temperature, may vary greatly in the ease with which it can be extracted. But in all cases, by sufficient repetition of the process, it is possible to render the acid-fast and Gram-positive bacteria completely non-acid-fast or Gram-negative.

The Quantities of "Lipoidal" Substances that can ve Removed from Various Bacteria.

Although the details of this subject still remain to be worked out, it is possible to state that the quantity of "lipoidal" substances obtainable with the formalin-acetone process from the tubercle bacillus is very much greater than that which can be removed from the non-acid-fast Gram-positive bacteria. The latter, in their turn, yield considerably more than the Gram-negative micro-organisms.

Again, the quantities obtainable from any given bacterium vary with the constitution of the medium on which it is grown, with the period of growth and the temperature of incubation.

The Microscopic Appearances of "Defatted" Antigen.

According to Salimbeni (1912) extraction of tubercle bacilli with trichlorhydrine rapidly makes them non-acid-fast and granular, and soon reduces them to an amorphous mass which does not take up the stain.

After extracting by the formalin-acetone process, the residue finally becomes completely non-acid-fast, and, on staining by ordinary methods, one finds that the bacillary character of the residue (*B. tuberculosis* antigen) has apparently been lost. The counterstain in Ziehl-Neelsen's method with ordinary aniline dyes merely shows faintly-stained amorphous masses. But, by means of prolonged staining with carbol-fuchsin, it is quite easy to demonstrate that the material really consists of bacilli, which stain too feebly to be demonstrated by the customary methods.

Technique.—A thin, uniform film of the antigen is made, dried, and fixed with ether-alcohol, followed by flaming.

An oblong piece of filter-paper is cut out so as to lie on the slide, covering the film, and extending nearly to the edges of the slide.

Carbol-fuchsin (Ziehl-Neelsen) is poured drop by drop on to the centre of the filter-paper, until this is thoroughly soaked all through. Air-bubbles between the filter-paper and the slide must be avoided. The slide is now warmed until steam rises, and then left to stain for 10 to 20 minutes.

Distilled water is then run on to the slide to wash off the stain and the filter-paper. The slide is then carefully washed until no more stain is given off. The film is then dried and examined in the usual way.

Demonstrated in this manner, the "defatted" tubercle bacilli appear as rods of extreme fineness and reduced length. Many of them are only faintly stained. Their average thickness is about that of the *Spirochæta pallida*, or even less. The leprosy bacillus undergoes a similar change. The degrees of reduction in size and of alteration in staining properties vary in the different groups of bacteria in proportion to the quantities of "lipoidal" substances that are removed. The non-acid-fast, Gram-positive bacteria undergo less profound alteration than the acid-fast group; they become Gram-negative, take the counterstain badly, and betray a noteworthy reduction in size. In the Gram-negative group the changes are the least marked of all, but even here are quite perceptible. The organisms become somewhat smaller and stain less intensely after extraction than before.

DIGESTIBILITY BY TRYPSIN OF "DEFATTED" BACTERIA.

It is generally accepted that proteins, after treatment with formaldehyde, cannot be digested with trypsin. Nevertheless all the "defatted" micro-organisms subjected to this test have proved to be digestible. Very dense suspensions can be almost entirely cleared with 1 per cent. trypsin (*injectio trypsini*, Fairchild).

The rate and completeness of the digestion, however, depend on a number of factors which remain at present undetermined, as, for instance, the amount of formalin retained in the proteins of the bacteria, the hydrogen-ion concentration, salt-content and so forth. It appears that very small alterations in these conditions can be responsible for very pronounced differences in the course of the reaction. One can, for instance, take the same "defatted" bacteria, grind them up in saline on two different occasions, add the same quantity of alkali and of trypsin, and maintain at the same temperature for the same length of time, and yet obtain, on the one occasion a perfect digestion, and on the other a relatively poor result, with only slight clarification.

These irregularities appear to be more pronounced with the tubercle-antigen than with the others, but they occur in all. At present, therefore, the preparation of a perfect digest is frequently a matter of considerable difficulty.

Preparation of Precipitinogens from "Defatted" Antigens.

The method used for the preparation of precipitinogen for the testing of a precipitating serum consists of the digestion of the "defatted" antigens with trypsin—a procedure based upon that employed by Sanarelli (1920), S. R. Douglas (1921) and others. Douglas showed that a precipitinogen resulted from the trypsin-cleavage of acetone-extracted Gram-negative bacteria.

Method.—The antigen-powder is ground up in sterile saline solution in an agate mortar to a dense suspension.

This is alkalised faintly, and from 1 to 2 per cent. of Fairchild's trypsin is

added. Digestion at 37° C. is now allowed to proceed for a period of not less than 2 hours and not more than 24. A more precise statement of the optimum time for digestion or of other optimal conditions is not at present possible.

As soon as it is thought that the digestion has proceeded far enough, the pH of the fluid is tested and brought back to the point at which it started.

It is at present uncertain whether it is best to heat for 10 mins. to 70° C. to destroy the trypsin or to leave it unheated. The fluid is now either centrifugalised or filtered through a coarse porcelain filter, whereupon it is ready for use.

Variability of digests.—Just as different specimens of defatted antigen vary in digestibility, so different digests of the same specimen of antigen, in spite of apparently identical conditions of preparation, show considerable variations in their sensitiveness as regards their reaction with precipitating sera. One digest may be highly sensitive, and another almost completely inert.

In this respect the time of digestion undoubtedly exercises an important influence, for a prolonged digestion, *e. g.* 30 hours, decreases the value of the resulting precipitinogen. The importance of other less defined factors remains for investigation, such as different hydrogen-ion concentration, salt-content, and a possible variation in the strength of the trypsin in different ampoules, due to varying chemical composition of the glass.

EXPERIMENTS WITH "DEFATTED" ANTIGENS.

Under this heading will be described experiments with a considerable variety of "defatted" antigens, arranged under headings of the various bacteria from which the antigens were made. The first and most prominent position is given to *B. tuberculosis*, because it is in many ways the most important of all, and because it was in connection with this organism that the idea of obtaining an adequately "defatted" antigen first arose. For this reason a more thorough study has so far been made of the tubercle-antigen than of any other.

EXPERIMENTS WITH B. TUBERCULOSIS.

I. Production of Immune Substances by Injection of "Defatted" Antigen into Normal Rabbits.

The "defatted" T.B. antigen referred to here, and throughout the paper, was made with a strain of *B. tuberculosis* known as "Lyons." The antigen contained 0·2 mgrm. of "defatted" bacilli per cubic centimetre.

Four rabbits, I, II, III, and IV, received the treatment shown in Table I.

As a result of the injections of "defatted" T.B. antigen these rabbits developed in varying degrees the following anti-substances, the presence of which in the serum was demonstrated by test-tube experiments—complement-fixing bodies, precipitins and agglutinins. The details will be found in the records and discussion of the *in vitro* experiments on pp. 157–159.

TABLE I

No. of Rabbit	Date	Sex	Weight	Dose of defatted antigen 1 cc = 0·2 mg	Route	Remarks
I	29·12·22	Female	2200	0·5 cc	Intravenous	
	4·1·23		2270	1·0 cc	"	
	17·3·23		2230	1·0 cc	"	12·2·23 had young all dead
	27·3·23		2200	1·0 cc	"	
	4·4·23		2420	1·0 cc	"	
	12·4·23		2555	1·0 cc	"	
	19·4·23		2050	1·0 cc	"	
	29·4·23		2040	1·0 cc	"	16·4·23 had young living
	18·5·23		2220	–	–	
II	29·12·22	Female	2150	0·5 cc	Intravenous	
	4·1·23		1950	1·0 cc	"	
	17·3·23		2390	1·0 cc	"	
	27·3·23		2670	1·0 cc	"	
	4·4·23		2620	1·0 cc	"	
	12·4·23		2590	1·0 cc	"	
	19·4·23		2550	1·0 cc	"	
	29·4·23		2800	1·0 cc	"	
	18·5·23		3190	–	–	
III	31·12·22	Male	2000	1·0 cc	Subcutaneous	No infiltration
	8·1·23		1970	1·0 cc	"	Small hæmatoma caused by injection. Gone in 2 days
	17·3·23		2370	1·0 cc	"	No infiltration
	27·3·23		2340	1·0 cc	Intravenous	
	4·4·23		2340	1·0 cc	"	
	12·4·23		2390	1·0 cc	"	
	19·4·23		2350	1·0 cc	"	
	29·4·23		2370	1·0 cc	"	
	18·5·23		2470	–	–	
IV	31·12·22	Male	2320	1·0 cc	Subcutaneous	No infiltration
	8·1·23		2320	1·0 cc	"	" "
	17·3·23		2350	1·0 cc	"	" "
	27·3·23		2410	1·0 cc	Intravenous	
	4·4·23		2500	1·0 cc	"	
	12·4·23		2520	1·0 cc	"	
	19·4·23		2500	1·0 cc	"	
	29·4·23		2620	1·0 cc	"	
	18·5·23		2600	–	–	

Technique of In Vitro *Tests.*

(A) *Complement-fixation.*

The experiments were carried out in dwarf test-tubes of about 1 cm. in diameter and 7 cm. in length, exactly similar to those employed as dilution-tubes in the agglutination technique.

Titration of complement.—Falling quantities of complement (*i.e.* normal guinea-pig serum) are added to fixed quantities of sensitised sheep's red cells and incubated for 30 minutes in the water-bath at 37° C. Thus:

Guinea-pig serum 1/20 .	10	9	8	7	6	5	4	3	2	1	0 drops.
0·9 per cent. saline solution	0	1	2	3	4	5	6	7	8	9	10 drops.
	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Sensitised sheep's red cells	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25 c.c.

N.B.—The saline is added to each tube in two stages—firstly, a sufficient number of drops to make serum *plus* saline always equal to 10 drops; secondly, 1 c.c. to each tube.

Titration of antigen.—Falling quantities of antigen are added to a fixed quantity of complement (2 minimal hæmolytic doses) and incubated for 1 hour in the water-bath at 37° C.; sensitised sheep cells are added, and the tubes are shaken and replaced in the bath for 1 hour.

The complement is diluted so that 5 drops contain 2 minimal hæmolytic doses. A control tube containing 15 drops antigen and no complement is included. Thus:

Complement	5	5	5	5	5	5	5	5	5	5 drops.
Antigen	15	12	10	8	6	5	4	3	2	1 „
Saline	0	3	5	7	9	10	11	12	13	14 „
	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Sensitised sheep's red cells	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25	0·25 c.c.

After 1 hour's incubation sensitised red cells are added, and the whole is reincubated for 1 hour at 37° C.

Test.—Falling quantities of the serum to be tested are added to fixed quantities of antigen and complement (2 M.H.D.) and incubated for 1 hour at 37° C.

Sensitised sheep cells are added. The tubes are shaken and replaced in the bath for 1 hour.

The antigen is diluted so that the largest dose which is not anti-complementary is contained in 15 drops.

The complement is diluted so that 2 hæmolytic doses are contained in 5 drops.

A control-tube to exclude anti-complementary action on the part of the test serum is included. Thus:

Tube	C (control)	1	2	3	4	5	6	7 etc.
Serum to be tested	5	5 (1:1)	2	1	10 (1:20)	5	2	1 drops.
Saline	20	5	8	9	0	5	8	9 „
	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Complement (2 M.H.D. per tube)	5	5	5	5	5	5	5	5 drops.
Antigen	0	15	15	15	15	15	15	15 „

The tubes are incubated for 1 hour at 37° C., after which 0·25 c.c. of sensitised sheep's red cells is added to each tube.

The tubes are shaken and replaced in the bath for 1 hour.

(B) *Precipitin reaction.*

This is carried out in the small pointed tubes known as Dreyer's agglutination tubes, either by adding 5 drops of the serum to each tube, and then 15 drops of a series of previously prepared graded dilution of antigen-digest, each dilution being double the previous one; or by adding falling quantities of serum to 15 drops of antigen-digest suitably diluted.

The results are read after incubation at 56° C. for 6 and 20 hours respectively.

(C) *Agglutination tests.*

These were carried out with the technique known as Dreyer's method, which is described in the Directions sent out by the Standards Laboratory, the University, Oxford, on behalf of the Medical Research Council.

II.—Production of Immune Substances in already Infected Rabbits with or without Injection of "Defatted" T.B. Antigen.

Rabbits V and VI were injected subcutaneously in the middle of the

TABLE II

RABBIT V, ♂ MALE, INJECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. (SEE TEXT). AT THE TIME OF THE FIRST INJECTION OF DEFATTED T.B. ANTIGEN THERE WAS A NODULE 2·0 cm. IN DIAMETER AT THE SITE OF INFECTION.

DATE	WEIGHT	DOSE OF DEFATTED ANTIGEN	ROUTE	REMARKS
12.1.23	2380	—	—	
18.2.23	2570	0·5 cc	SUBCUTANEOUS	24 HOURS AFTER THIS INJECTION A WELL MARKED HARDISH INFILTRATION APPEARED WHICH GRADUALLY DIMINISHED, AND HAD DISAPPEARED ON THE 5TH DAY.
8.3.23	2600	0·5 cc	INTRAVENOUS	
18.3.23	2390	1·0 cc	"	
28.3.23	2390	0·5 cc	"	
4.4.23	2600	0·5 cc	"	
12.4.23	2500	0·5 cc	"	
19.4.23	2400	0·5 cc	"	
29.4.23	2720	0·75 cc	"	
18.5.23	2820	—	—	

TABLE III

RABBIT VI - FEMALE, INJECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. ON 15.12.23 IT HAD A NODULE ABOUT 1·5 cm IN DIAMETER AT SITE OF INFECTION.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2400	–	–	
15.2.23	2690	0·5 cc	SUBCUTANEOUS	MARKED INFILTRATION AT SITE OF INJECTION AFTER 24 HOURS, WHICH SLOWLY DECREASED BUT DID NOT ENTIRELY DISAPPEAR TILL THE 10TH DAY
8.3.23	2800	0·5 cc	INTRAVENOUS	
17.3.23	2600	1·0 cc	"	
27.3.23	2800	0·5 cc	"	
4.4.23	2750	0·5 cc	"	HAD LITTER OF YOUNG ON 3.4.23.
12.4.23	2850	0·5 cc	"	
19.4.23	2850	0·5 cc	"	
29.4.23	2890	0·75 cc	"	
18.5.23	3030	–	–	

abdomen on 12.i.23 with 0·5 c.c. of a suspension of a strain of a living bovine tubercle bacillus (Pasteur Institute), here referred to as "A." The suspension contained about 200 to 300 million bacilli per c.c.

TABLE IV.

RABBIT VII - FEMALE, INFECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. (SEE TEXT). AT TIME OF FIRST ANTIGEN INJECTION THERE WAS A NODULE ABOUT 1·5 cm IN DIAMETER AT SITE OF INFECTION.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2200	–	–	
15.2.23	2540	0·5 cc	SUBCUTANEOUS	LARGE SWELLING WITH HYPERÆMIA AFTER 24 HOURS DECREASED GRADUALLY, DISAPPEARING ENTIRELY ON THE 7TH DAY.
8.3.23	3050	0·5 cc	INTRAVENOUS	
17.3.23	2450	1·0 cc	"	
28.3.23	2550	0·5 cc	"	
4.4.23	2840	0·5 cc	"	
12.4.23	2940	0·5 cc	"	HAD YOUNG. 13.4.23.
19.4.23	2700	0·5 cc	"	
29.4.23	3050	0·75 cc	"	
18.5.23	2920	–	–	HAD YOUNG. ALL DEAD.

TABLE V.

RABBIT VIII - MALE, INFECTED WITH LIVE TUBERCLE BACILLI ON 12.1.23. ON 15.2.23 THERE WAS A NODULE OF ABOUT 2·0 cm IN DIAMETER AT SITE OF INFECTION BREAKING THROUGH SKIN, AND A GLAND IN THE RIGHT AXILLA THE SIZE OF A FILBERT NUT.

DATE	WEIGHT	DOSE	ROUTE	REMARKS
12.1.23	2600	–	–	–
15.2.23	2850	–	–	–
8.3.23	3070	–	–	NODULE OPEN, ULCERATING.
17.3.23	3040	–	–	–
27.3.23	2850	–	–	–
4.4.23	2900	–	–	LARGE AXILLARY GLAND.
12.4.23	2840	–	–	–
19.4.23	2800	–	–	ULCERATION OF ABOUT 2·5 cm OVER NODULE
29.4.23	2980	–	–	–
18.5.23	2540	–	–	ULCER UNCHANGED; GLAND SLIGHTLY LARGER

Rabbits VII and VIII received on 12.i.23 a subcutaneous abdominal injection of 0·5 c.c. of a suspension of another strain of a living bovine tubercle

bacillus (Pasteur Institute) here referred to as "B." This suspension was thinner, containing only about 100–200 million bacilli per c.c.

The production of immune substances in these animals and in the normal animals previously referred to was as follows:

III.—The Character of the Immune Substances Developed.

Complement-fixation Tests.

From Table VI it is clear that so far as complement-fixation is concerned the "defatted" T.B. antigen *is specific* in its reaction, not only with the serum derived from a horse immunised with whole tubercle bacilli, but also with the serum of a rabbit suffering from tuberculosis.

As *controls* of the test of the anti-tuberculous horse-serum, strong anti-diphtheritic sera were used, in order to ascertain whether the serum of an animal which had developed a high immunity to a totally different antigen gave any reaction with the "defatted" tubercle bacilli. This is clearly not the case.

TABLE VI

DESCRIPTION OF SERUM	QUANTITY OF SERUM IN DROPS*	RELATIVE STRENGTH OF SERA
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/10	500
ANTIDIPHTHERITIC HORSE-SERUM-400 UNITS	>5	<1·0
ANTIDIPHTHERITIC HORSE-SERUM-500 UNITS	5	1·0
RABBIT INJECTED WITH LIVING BOVINE B. TUBERCULOSIS	1/2	10·0
NORMAL RABBIT 1.	5	1·0
NORMAL RABBIT 2.	5	1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

The "defatted" antigen having proved to be specific, the next step was to investigate the complement-fixation reactions of normal animals injected with "defatted" T.B. antigen, and of rabbits suffering from tuberculosis, some of which had been treated with "defatted" antigen, while others had not.

TABLE VII

DESCRIPTION OF SERUM		QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERA
NORMAL RABBITS INJECTED WITH DEFATTED T.B. ANTIGEN	No I	1	5·0
	II	2	2·5
	III	2	2·5
	IV	3	1·7
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T.B. ANTIGEN	V	1	5·0
	VI	1/4	20·0
	VII	1/4	20·0
RABBIT INJECTED WITH LIVING T.B. NO SUBSEQUENT TREATMENT	VIII	1	5·0
NORMAL RABBITS	A	>5	<1·0
	B	>5	<1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

Table VII shows that the injection of "defatted" antigen into normal rabbits gives rise to complement-fixing substances. On the whole, the infected animals, subsequently injected with "defatted" antigen, seemed to give stronger reactions than either those that were simply infected, or the normals that had received "defatted" antigen. But it is impossible to draw a definite conclusion on this point from such a small number of experiments.

It was furthermore of interest to compare the activity of the "defatted"

TABLE VIII

COMPARISON BETWEEN DEFATTED T.B. ANTIGEN AND THE ANTIGEN OF BOQUET & NÈGRE

DESCRIPTION OF SERA		DEFATTED T.B. ANTIGEN: QUANTITY OF SERUM IN DROPS *	DEFATTED T.B. ANTIGEN: RELATIVE STRENGTH OF SERA	ANTIGEN OF BOQUET & NÈGRE: QUANTITY OF SERUM IN DROPS *	ANTIGEN OF BOQUET & NÈGRE: RELATIVE STRENGTH OF SERUM
NORMAL RABBITS INJECTED WITH DEFATTED T. B. ANTIGEN	No I	1/2	10·0	1	5·0
	II	1/2	10·0	7	0·7
	III	2	2·5	8	0·6
	IV	5	1·0	6	0·8
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T. B. ANTIGEN	V	1	5·0	6	0 8
	VI	1/4	20·0	7	0 7
	VII	1/2	10·0	2	2·5
RABBIT INJECTED WITH LIVING T. B. NO SUBSEQUENT TREATMENT	VIII	1	5 0	1	5·0
NORMAL RABBITS	A	7	0·7	7	0·7
	B	8	0·6	8	0·6

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GIVES COMPLETE COMPLEMENT-FIXATION.

antigen with that of a recognised antigen of the usual type, and for this purpose Boquet's and Nègre's antigen was chosen. From Table VIII it is evident that the "defatted" antigen in the majority of cases shows by far the greater activity of the two.

TABLE IX

FALLING QUANTITIES OF SERUM. FIXED QUANTITY OF DIGEST OF DEFATTED ANTIGEN.

DESCRIPTION OF SERUM		HIGHEST DILUTIONS OF SERUM SHOWING EQUAL DEGREES OF PRECIPITATION	RELATIVE ACTIVITY OF SERA
RABBITS INFECTED WITH LIVING T. B. AND SUBSEQUENTLY INJECTED WITH DEFATTED ANTIGEN	No V	1/25	10·0
	VI	1/12·5	5·0
	VII	1/25	10·0
RABBIT INFECTED WITH LIVING T. B. NO OTHER TREATMENT	VIII	1/50	20·0
NORMAL RABBITS	A	>1/2·5	<1·0
	B	>1/2·5	<1·0

TABLE X

FIXED QUANTITY OF SERUM. GRADED DILUTIONS OF DIGEST OF DEFATTED ANTIGEN.

		HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION	RELATIVE ACTIVITY OF SERA
RABBITS INJECTED WITH DEFATTED T. B. ANTIGEN	No I	1/170	5·3
	II	1/42	1·3
	III	1/64	2·0
	IV	1/128	4·0
RABBITS INFECTED WITH LIVING T.B. THEN INJECTED WITH DEFATTED T.B. ANTIGEN	V	1/256	8·0
	VI	1/128	4·0
	VII	1/128	4·0
RABBIT INFECTED WITH LIVING T.B. NO OTHER TREATMENT	VIII	1/128	4·0
NORMAL RABBITS	A	1/32	1·0
	B	1/32	1·0

Precipitin Reactions.

These are shown in Tables IX and X.

In Table IX it is seen that the T.B. precipitinogen obtained by digesting "defatted" T.B. with trypsin is *specific* in its reaction. This corresponds with S. R. Douglas's experience in the case of acetone-extracted Gram-negative bacteria. The sera of animals suffering from tuberculosis, whether injected with defatted antigen or not so treated, give precipitation with the digest. That the injection of defatted T.B. antigen into normal rabbits gives rise to specific precipitins is shown in Table X. As in the case of complement-fixation, there is here also an indication that the highest reactions are given by animals which have been infected with the tubercle bacillus, and subsequently treated with "defatted" antigen.

Agglutination Experiments.

Tables XI and XII illustrate the agglutination experiments, which were performed with saline suspensions of the "defatted" bacilli diluted to a suitable constant opacity with normal saline formolised to 0·1 per cent.

Table XI shows that the sera of tuberculous animals contain agglutinins which react *specifically* with the "defatted" bacilli.

From Table XII we can conclude that the injection of "defatted" antigen into normal rabbits causes the production of specific agglutinins, and that these substances are also present in the serum of animals infected with the tubercle bacillus whether they have or have not received subsequent injections of "defatted" antigen. Once more the combination of infection with injection seems to yield, on an average, the strongest sera.

TABLE XI

DESCRIPTION OF SERA	HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION	RELATIVE ACTIVITY OF SERA
HORSE IMMUNIZED WITH TUBERCLE BACILLUS	1/100	40·0
HORSE ANTI-ANTHRAX SERUM (MULFORD)	> 1/2·5	< 1·0
TUBERCULOUS RABBIT	1/12·5	5·0
NORMAL RABBIT	> 1/2·5	< 1·0

TABLE XII

DESCRIPTION OF SERA		HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION	RELATIVE ACTIVITY OF SERA
NORMAL RABBITS INJECTED WITH DEFATTED T.B. ANTIGEN	No I	1/250	100·0
	II	1/250	100·0
	III	1/50	20·0
	IV	1/25	10·0
RABBITS INFECTED WITH LIVING T.B. AND SUBSEQUENTLY INJECTED WITH DEFATTED T.B. ANTIGEN	V	1/100	40·0
	VI	1/500	200·0
	VII	1/250	100·0
RABBIT INJECTED WITH LIVING T.B. NO SUBSEQUENT TREATMENT	VIII	1/100	40·0
RABBIT INJECTED WITH FATTY EXTRACT OF T.B. EMULSIFIED IN SALINE	23	> 1/2·5	< 1·0
NORMAL RABBITS	X	1/5	2·0
	Y	1/2·5	1·0

IV. Treatment with "Defatted" T.B. Antigen of Animals Infected with *B. tuberculosis.*

The experiments detailed in this section were performed with a view to studying the therapeutic effects of "defatted" tubercle-antigen on animals suffering from well-defined tuberculosis.

Guinea-pigs.

Four animals have been thoroughly studied, *i. e.* Nos. 4, 26, 27 and 28.

Nos. 26, 27 and 28 were kindly given to me by Capt. S. R. Douglas, with the object of accelerating my results. Nos. 27 and 28 had been infected with *B. tuberculosis* by Capt. Douglas on April 20th, 1922. No. 26 had been similarly infected, but neither the exact date of infection nor the weight of the animal at the time can be traced. The infection, however, took place several months before treatment was begun. Guinea-pig 4 was infected in my laboratory on June 15th, 1922, with the same strain of tubercle bacillus as had been used by Douglas.

The bacillus used, "T.B. 100," is the strain used at the Höchst laboratory for making tuberculin. Douglas received it from Schottelius in 1908. It is a bacillus of the human type, and has never passed through an animal since it has been in Douglas's possession. He has injected at least 50 guinea-pigs with this strain during the last two years. The culture is, on the whole, of slight virulence. On good media it grows well, and definite growth may be obtained

in 3 to 4 days. The infected animals have usually died in 4 to 10 months (most commonly 6 to 8 months) after infection, and the *post-mortem* examination has invariably shown a generalised tuberculosis, with the following features: *Spleen* enlarged, with sago granules, but no large areas of disease. *Liver* somewhat enlarged, with similar granules. *Lungs* showing pinhead tubercles. *Lymph-glands*: the axillary and cervical glands showed well-marked enlargement (size of a pea or more); the inguinal and lumbar glands always enlarged and caseous.

In smears of the spleen and lymph-glands the tubercle bacillus could always readily be demonstrated in considerable numbers, and cultures were easily obtained.

The animals 26, 27 and 28 were the last survivors of a batch of 25 that were injected on April 20th, 1922. At the time of injection most of them weighed between 400 and 500 grm. Many of this batch were healthy survivors from the testing of diphtheria antitoxin.

At the time when treatment was started all animals showed well-marked signs of tuberculosis. The details of the condition of the animal and of the injections will be found under each animal.

All the animals were weighed every day from the beginning of treatment, but the weights given in the tables are averages of 7 days' weighing.

Guinea-pig No. 4 (male). (See Table XIII and Chart I.)

Condition before treatment.—Treatment was begun on 29 . xii . 22, 28 weeks after the animal had been infected with T.B. 100. The animal looked

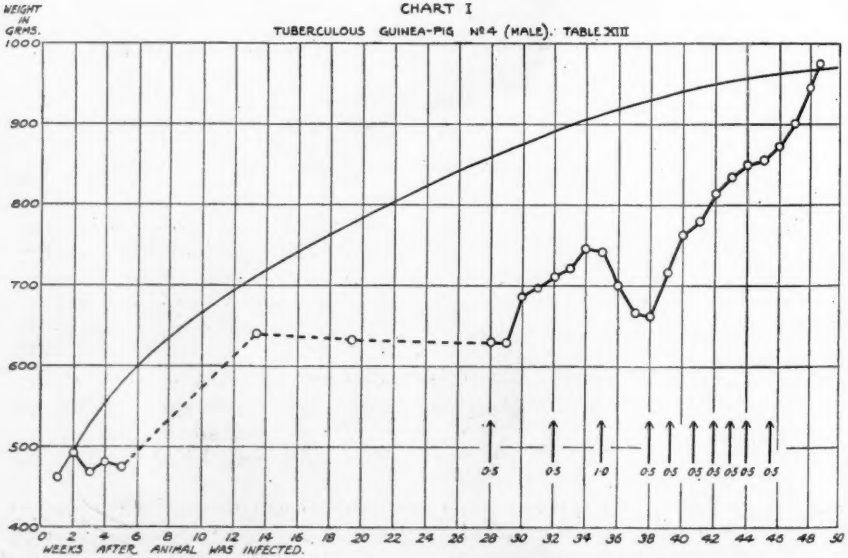


CHART I
TUBERCULOUS GUINEA-PIG Nº4 (MALE). TABLE XIII
WEIGHT IN GRMS.
1000
900
800
700
600
500
400
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
WEEKS AFTER ANIMAL WAS INFECTED.
0·5
0·5
1·0
0·5 0·5 0·5 0·5 0·5 0·5
N.B. A CURVE OF THE NORMAL PROGRESS IN WEIGHT OF ANIMALS OF THE SAME AGE AS G.P. 4, CALCULATED FROM 6 HEALTHY ANIMALS, IS INCLUDED FOR COMPARISON.

TABLE XIII.

TUBERCULOUS GUINEA-PIG Nº 4 (MALE).

DATE	AVERAGE WEEKLY WEIGHT *	WEEKS FROM INFECTION	INJECTIONS DATE & DOSE	REMARKS
21.6.22	462	1		
28.6.22	490	2		
5.7.22	468	3		
12.7.22	480	4		
19.7.22	475	5		
16.9.22	640	13 3/7		
				21.10.22 TESTED WITH OLD TUBERCULIN GRADED VON PIRQUET STRONGLY POSITIVE.
28.10.22	633	18 3/7		
28.12.22	630	28		
			29.12.22 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 10 DAYS.
4.1.23	629	29		
11.1.23	685	30		
18.1.23	696	31		
			24.1.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 6 DAYS.
25.1.23	710	32		
1.2.23	720	33		
8.2.23	746	34		
			15.2.23 1·0cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 13 DAYS.
15.2.23	740	35		
22.2.23	700	36		
1.3.23	666	37		
			8.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 3 DAYS.
8.3.23	661	38		
15.3.23	716	39		
			17.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 7 DAYS.
22.3.23	763	40		
			27.3.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
29.3.23	780	41		
			4.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
5.4.23	815	42		
12.4.23	836	43	12.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 4 DAYS.
19.4.23	850	44	19.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 5 DAYS.
26.4.23	856	45		
			29.4.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 4 DAYS.
3.5.23	874	46		
10.5.23	906	47		
17.5.23	946	48		

* I.E. AVERAGE OF 7 DAYS WEIGHING, ENDING ON DATE GIVEN.

healthy, but was under the average weight for its age, scaling 630 grm. instead of 800 to 900 grm. The spleen was large and could easily be felt. There were 3 or 4 enlarged glands in the right inguinal region, and 1 or 2 on the left side. Those on the right were about the size of a large pea, those on the left the size of a small pea. There was diffuse infiltration around the glands.

Condition after treatment (48 weeks after infection).—The animal looks perfectly healthy, and is now of the normal weight for its age. The spleen seems smaller. The inguinal glands on the right side are smaller and harder. The glands on the left side have practically disappeared, being replaced by what feels like a pad of fibrous tissue.

In an animal of this species the weight is the best indication of the general condition of health (see Table XIII and Chart I). In the absence of treatment this animal would have been expected to die within 10 months from the date of infection.

Guinea-pig No. 26 (male). (See Table XIV and Chart II.)

Condition before treatment.—The animal looked healthy. The spleen was large and could easily be felt. There were large masses of glands in the right

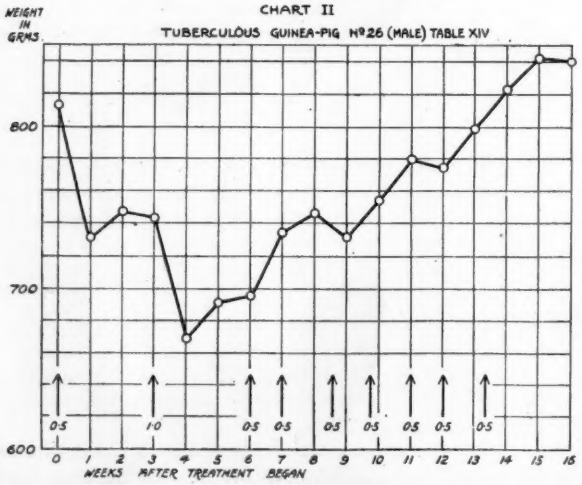
TABLE XIV

TUBERCULOUS GUINEA-PIG No 26 (MALE)

DATE	AVERAGE WEEKLY WEIGHT	WEEKS AFTER COMMENCEMENT OF TREATMENT	INJECTIONS DATE & DOSE	REMARKS
26.1.23	813	0	24.1.23 0·5cc	INJECTION FOLL. BY A VERY WELL-MARKED INFILTRATION LASTING 11 DAYS.
2.2.23	731	1		
9.2.23	747	2		
			15.2.23 1·0cc	INJECTION FOLL. BY A VERY LARGE INFILTRATION LASTING 14 DAYS.
16.2.23	743	3		
23.2.23	669	4		
2.3.23	691	5		
			8.3.23 0·5cc	INJECTION FOLL. BY INFILTRATION LASTING 3 DAYS.
9.3.23	695	6		
16.3.23	736	7		
			17.3.23 0·5cc	INJECTION FOLL. BY SMALL INFILTRATION LASTING 4 DAYS.
23.3.23	746	8		
			27.3.23 0·5cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
30.3.23	731	9		
			4.4.23 0·5cc	INJECTION FOLL. BY NO INFILTRATION.
6.4.23	754	10		
13.4.23	779	11	13.4.23 0·5cc	INJECTION FOLL. BY NO INFILTRATION.
			19.4.23 0·5cc	INJECTION FOLL. BY VERY SLIGHT INFILTRATION LASTING 4 DAYS.
20.4.23	774	12		
27.4.23	799	13		
			29.4.23 0·5cc	INJECTION FOLL. BY ?INFILTRATION LASTING 2 DAYS.
4.5.23	823	14		
11.5.23	842	15		
18.5.23	840	16		

CHART II

TUBERCULOUS GUINEA-PIG No 26 (MALE) TABLE XIV



and left inguinal regions, most marked on the right. About 2 months before treatment began the animal gave a positive von Pirquet reaction (graded).

Condition after treatment.—The animal looked healthy. Its weight is shown in Chart II. The glands in both inguinal regions had already entirely disappeared by 23 . iii . 23, *i.e.* 8 weeks after treatment began. On 18 . v . 23 no sign of any glands remained, though there was perhaps a slight residual thickening of the subcutaneous tissue.

Guinea-pig 28 (male). (See Table XV and Chart III.)

Condition before treatment.—Treatment was begun on 24 . i . 23, 39 weeks after infection with T.B. 100. The animal looked thin and poorly. It was

TABLE XV.

TUBERCULOUS GUINEA-PIG № 28 (MALE).

DATE	AVERAGE WEEKLY WEIGHT	WEEKS AFTER INFECTION	INJECTIONS DATE & DOSE	REMARKS
26 . 1 . 23	542	39	24 . 1 . 23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 5 DAYS.
2 . 2 . 23	508	40		
9 . 2 . 23	513	41		ANIMAL ILL, WITH PURULENT SECRETION FROM NOSE, LASTING TILL 28 . 2 . 23.
			15 . 1 . 23 1·0 cc	ANIMAL ILL, WITH LARGE INFILTRATION LASTING 5 DAYS.
16 . 2 . 23	514	42		
23 . 2 . 23	506	43		
2 . 3 . 23	503	44		ANIMAL HAS RECOVERED FROM ITS INTERCURRENT ILLNESS.
			8 . 3 . 23 0·5 cc	INJECTION FOLL. BY INFILTRATION LASTING 2 DAYS.
9 . 3 . 23	524	45		
16 . 3 . 23	572	46	17 . 3 . 23 0·5 cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 3 DAYS.
23 . 3 . 23	599	47		
30 . 3 . 23	578	48	27 . 3 . 23 0·5 cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 3 DAYS.
			4 . 4 . 23 0·5 cc	INJECTION FOLL. BY SLIGHT INFILTRATION LASTING 5 DAYS. ANIMAL ILL AGAIN WITH PURULENT SECRETION FROM NOSE, WHICH PERSISTED IN DIMINISHING DEGREE TILL 18 . 5 . 23.
6 . 4 . 23	595	49		
			12 . 4 . 23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 6 DAYS.
13 . 4 . 23	549	50		
20 . 4 . 23	556	51	20 . 4 . 23 0·5 cc	INJECTION FOLL. BY MARKED INFILTRATION LASTING 7 DAYS.
27 . 4 . 23	567	52		
			29 . 4 . 23 1·0 cc	INJECTION FOLL. BY LARGE INFILTRATION LASTING 8 DAYS. ANIMAL LOOKED THIN AND ILL, AND HAD DIARRHŒA WHICH WAS STILL PRESENT, THOUGH MUCH LESS ON 15 . 5 . 23. A SHARP DROP OF WEIGHT STARTED ON 52ND WEEK.
4 . 5 . 23	494	53		
11 . 5 . 23	525	54		
18 . 5 . 23	508	55		THE ANIMAL LOOKS MUCH BETTER, BUT STILL HAS SOME DIARRHŒA.

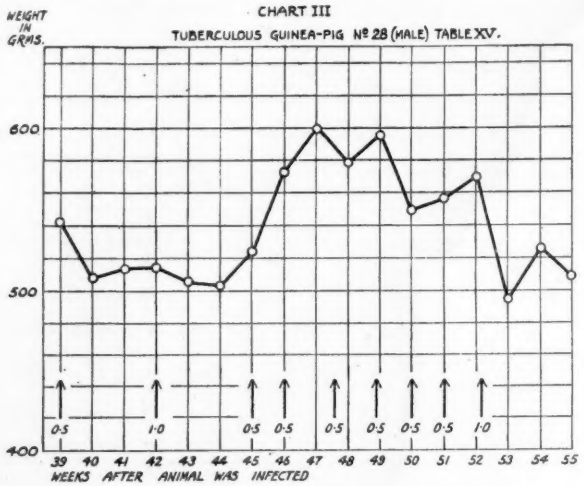
greatly under weight, scaling only 540 grm. instead of 800–900 grm., which would have been the average normal weight of an animal of that age. The spleen was large and easily felt.

There were four or five large lymph-glands in right inguinal region and a similar number of still larger ones on the left side. One gland had ulcerated through the skin some 4 weeks before treatment. The ulcer was unhealthy looking and discharged caseous matter.

Condition during treatment.—Four days after the 1st injection the ulcer appeared smaller and clean. The discharge had ceased.

Ten days after the 1st injection the ulcer had healed up, and showed a nice smooth scar.

Within 24 hours of the 2nd injection the scar broke down, showing a healthy raw surface about 1 cm. long, without any discharge. It healed again within a week of the injection. Meanwhile the glands on both sides had definitely diminished in size.



After the 3rd injection the scar again opened in about 66 hours. It was fully healed again in 6 days.

Twenty-four hours after the 4th injection the same thing occurred, and healing was complete in another 5 days.

After the 5th injection the scar remained healed. After the 6th it broke down slightly, to heal again in 3 days.

The 7th injection caused a tiny opening of the scar, which persisted 4 days.

On the 8th injection no breaking down occurred. Meanwhile a steady decrease in the size of the glands was in progress.

After the 9th injection the scar held firm.

Condition on 18 . v . 23.—The glands are greatly reduced in size. The ulcer is replaced by strong, healthy scar-tissue. The animal weighs a little over 500 grm. (see Chart III).

Guinea-pig No. 27 (female). (See Table XVI.)

Condition before treatment.—Treatment began on 24 . i . 23, 39 weeks after infection. The animal looked poorly. It was greatly under weight (550 grm. instead of a normal weight of about 800 grm.). The spleen was

large and easily palpable. There was a mass of inguinal glands with diffuse periadenitis, which obscured the individual glands. No ulceration.

Condition during treatment.—The animal seemed to improve in appearance and the glands gradually diminished in size, becoming hard and discrete. The periadenitis had largely disappeared.

After the birth of its young the animal suddenly started to go downhill and died in a few days. It is impossible to state the cause of death with certainty. The heart-blood, cultivated in broth and on agar, remained sterile.

TABLE XVI

TUBERCULOUS GUINEA-PIG No 27 (FEMALE)

DATE	AVERAGE WEEKLY WEIGHT	WEEKS FROM INFECTION	INJECTIONS DATE & DOSE	REMARKS
26.1.23	553	39	24.1.23 0·5 cc	MARKED INFILTRATION LASTING 11 DAYS.
2.2.23	540	40		
9.2.23	573	41		
			15.2.23 1·0 cc	LARGE INFILTRATION LASTING 9 DAYS.
16.2.23	567	42		
23.2.23	547	43		
2.3.23	543	44		
6.3.23				NOTICED TO BE PREGNANT
			8.3.23 0·5 cc	INFILTRATION LASTING 3 DAYS.
9.3.23	569	45		
16.3.23	649	46		
			17.3.23 0·5 cc	LARGE INFILTRATION LASTING 5 DAYS.
23.3.23	709	47		
			27.3.23 0·5 cc	SLIGHT INFILTRATION LASTING 3 DAYS.
30.3.23	717	48		
			4.4.23 0·5 cc	TRACE OF INFILTRATION LASTING 1 DAY.
6.4.23	726	49		
7.4.23				HAD YOUNG. 2 DEAD, 1 LIVING.
8.4.23				ANIMAL SUDDENLY ILL. LOST 100 grms. IN
11.4.23	438	(50)		NEXT 3 DAYS.
12.4.23				FOUND DEAD.

Since this animal was a female, and became pregnant, a graph of the weight would be of no interest.

Dr. A. G. Gibson kindly undertook to examine the organs of this guinea-pig, and reports as follows :

Guinea-pig No. 27 (female) ; *naked-eye appearances.*

The *inguinal glands* are enlarged to about the size of a lentil, with surrounding fibrosis, which is reddish. There is no caseation seen on section.

The *axillary glands* are enlarged ; one on the left side is the size of a pea.

The *spleen* is much enlarged, 5 by 2·5 cm. The surface is pale and anæmic with areas of congestion of irregular outline. These areas probably constitute what remains of normal spleen. Under the capsule can be seen dots suggesting miliary tubercles. On section the congested and anæmic areas are seen as on the surface. The pulp is firm, and the white areas suggest the consistency of a pale infarct. Neither the Malpighian bodies nor the trabeculæ can be distinguished.

The *liver* is diminished in size. The surface is irregular, especially that of the middle and left lobes. Three types of structure can be discerned :

1. Yellowish or greenish-yellow areas 3-4 mm. across, suggesting abscesses.

2. Anæmic areas suggesting granulomata, varying in size from that of a small pin's head to an area 6–8 mm. across.

3. Linear and radiate depressions—the result of shrinkage of liver-tissue. The rest of the liver shows congestion and fibrosis and in parts there is the appearance of hæmorrhage. The capsule is not thickened. On section the same three lesions are evident. The first contains semi-solid greenish necrotic substance. The second is pale, and suggests recently-formed fibrous tissue. The third suggests scarring as in human disease from the healing of an infarct or gumma.

Externally the *lungs* are congested, but show no evidence of gross disease, either externally or on section.

The *supra-renals* and *kidneys* are normal.

The *peritoneum*, both the *pleuræ* and the *pericardium*, show no lesions.

Microscopic Appearances.

Gland 1.—The section stained with hæmatoxylin and eosin shows two lymph-glands embedded in fatty tissue, with hyperæmia and some increase of cells. The gland itself shows both large and small areas unstained except for a variable number of nuclei. The gland-tissue has almost disappeared except at one end, and here and there in other parts. The pale area shows the ghosts of cells and nuclei, and in the centre of one there is a badly-stained giant-cell. The appearance of these ghost-like areas suggests healing tubercles that have subsequently undergone anæmic necrosis. The cells are of the endothelioid type, widely spaced from one another. Stained by van Gieson (Hansen's modification) these areas show abundance of collagen fibres giving them a redder appearance than the neighbouring parts of the gland. Practically the whole of the gland-tissue shows diffuse fibrosis, which is especially intense near the capsule. The only parts free from this change are the remains of the normal gland.

There is no evidence of necrosis or of caseation as is usually seen in tuberculous glands. Some pigmentation is present.

Gland 2.—Agrees in general features with No. 1, except that the pigmentation is more marked. The pale areas that lack full nuclear staining again show definite fibrosis. There is no evidence of necrosis or caseation, except for the appearances described. In the more cellular zone outside the pale areas there are fragments of nuclei but nothing that could be taken for necrosis, for side by side with these fragments there are complete and well-stained nuclei.

The *liver*, stained with hæmatoxylin and eosin, shows in every section granulomata, some highly cellular, and others less so. The first type shows fragments of nuclei, endothelioid cells, lymphocytes, and a few ill-formed giant-cells, but no evidence of necrosis or caseation. In the less cellular there is an eosin-staining matrix, in which can be made out a cellular structure suggesting liver-tissue. Here and there are nuclei, some ill-formed and irregular; some of these appear to be the lining cells of a capillary. These areas suggest infarction. The general lymphocytic reaction is small, except here and there, and some of the areas are full of fibroblasts with

poorly-stainly nuclei. The liver-tissue elsewhere shows large fat-globules through the lobules. In Glisson's capsule there is seen proliferation of the bile-ducts. This is very marked in one section. Van Gieson's stain (Hansen's modification) shows a marked increase of fibrous tissue everywhere, but particularly of Glisson's capsule. The granulomata for the most part show a peripheral zone of fibrous tissue, which in some cases seems to be spreading inwards. Fibrous tissue is not present in the area that shows infarction. There is much congestion of the liver-tissue in parts. A marked increase in the number of bile-ducts is seen in one part.

The *lungs*, stained with *hæmatoxylin and eosin*: Abnormal areas are present in the lungs, involving the connective tissue accompanying the artery and bronchus, and invading the lung-tissue in the neighbourhood. The alveoli here are largely obliterated, the septa are thickened and the alveoli and bronchioles contain catarrhal cells. Only here and there is there some round-celled lymphocytic infiltration. The *van Gieson-stained* section shows in these areas early general fibrosis, most intense in the peri-arterial tissues. Fibrous tissue is penetrating the whole thickness of the bronchial wall. Elsewhere the lung shows slight catarrh and congestion.

The *spleen*, stained with hæmatoxylin and eosin: The large pale areas appear as patches of anæmic necrosis, the cellular structure being ill-defined by reason of faulty staining of nuclei. Careful examination, however, reveals a granulomatous condition, with here and there large giant cells, multi-nucleate, the nuclei occasionally arranged in a semi-circle, staining poorly with hæmatoxylin. The rest of the nuclei appear to be those of feebly staining endothelial cells and of the few lymphocytes with normal staining properties in the periphery of the pale areas, and in a few other spots. The spleen tissue is very hyperæmic, with large blood spaces. A few miliary tubercles in various stages are seen, some with necrosis in the centre. Stained by van Gieson's method (Hansen's modification) Malpighian bodies are not recognisable, and there is little evidence of other normal appearances. Very few arteries can be distinguished. Two cross-sections of a medium-sized vessel show no change. The structure is so obscured that the central artery of the Malpighian bodies can in no case be certainly recognised.

The fibrous tissue is abundant in the altered splenic tissue which separates the areas of poor or absent nuclear staining. In some instances these latter areas are surrounded by a ring of fibrous tissue which penetrates some distance towards the centre, but this penetration and replacement is not so complete or marked as in the glands, where the tubercles are now completely fibrous.

The poorly staining areas with few nuclei correspond to the anæmic areas seen with the naked eye. The structure indicates death of tissue; it is not, however, caseous, nor is it the necrotic tissue with disintegrated nuclei that is commonly seen in the active parts of tuberculous lesions. It recalls most completely the anæmic necrosis seen in infarction, with this difference—that the necrotic tissue, instead of showing faintly the structure of the normal organ, here shows that of a tuberculous lesion, with recognisable endothelial cells and giant-cells. The paucity of vessels may be recalled in this connection.

Summary.—The lesions in all the organs are tuberculous granulomata.

The necrotic areas are neither caseation nor the necrosis with karyorrhexis seen in tuberculous lesions, but a gross anæmic necrosis more often seen in infarction. This anæmic necrosis however, appears to be superimplanted upon a tuberculous lesion. All the lesions, especially those in the liver, show abundant fibrosis, and throughout there is an absence of the lymphocytic reaction seen in active tuberculosis. The presence of giant-cells, together with the increase of fibrous tissue, lend support to the evidence which points to a tendency to heal. In sections of the spleen, liver, lungs and lymphatic glands only a very few tubercle bacilli can be found, *i.e.* some 4 to 12 bacilli per section.

Rabbits.

Four rabbits, 5, 6, 7 and 8, were infected on 12 . i . 23 with living bovine *B. tuberculosis.*

Three of these underwent a course of treatment with " defatted " tubercle-antigen, while the fourth served as a control. Treatment was begun on 15 . ii . 23, *i.e.* 5 weeks after the infection, and up to the present it has extended over three months. The results, which are not yet final, will be given in the form of a short summary of the condition of each animal at the beginning and at the end of the period.

Details of the doses of " defatted " antigen, of the reactions of the animals, their weights, etc., will be found in Tables II, III, IV and IV.

Rabbit 5. (Table II.)

Before treatment.—A prominent nodule was present at the site of infection, and there were some slightly enlarged glands in the left inguinal region.

After treatment.—Both the glands and the nodule had disappeared. The weight had increased considerably.

Rabbit 6. (Table III.)

Before treatment had a well-developed nodule at site of infection, but showed no enlarged lymph-glands.

After treatment the nodule was much reduced in size and felt hard, like fibrous tissue. Its size and characters had not altered appreciably for 3 weeks. The animal's weight underwent considerable increase during treatment.

Rabbit 7. (Table IV.)

Before treatment.—Showed a nodule of moderate size at the site of injection, and also some small inguinal glands.

After treatment.—The nodule had almost completely disappeared, but the glands could still be felt. They were small and hard, and had shown very little change for two weeks past. The animal's weight had increased greatly.

Rabbit 8. (Table V.)

At the beginning of the period.—The infection nodule of this animal had ulcerated through the skin, and it had a large axillary gland.

At the end of the period.—The nodule and the ulcer were stationary. without any tendency to heal. The animal's weight had not increased. At first there was an increase, but then the weight fell again to its original level.

Summary of the Results of Treatment of Animals with "Defatted" *B. tuberculosis* Antigen.

The experiments just described justify, in my opinion, the conclusion that treatment with the "defatted" antigen brings about a definite improvement, both general and local, in animals infected with *B. tuberculosis.* Additional evidence for this view is given by the guinea-pig No. 27 which died, for its organs show what seems indubitably to be a healing of tuberculous lesions.

Of course only time can show whether this beneficial effect is permanent. In order to attain certainty on this question, it will be necessary to watch the animals for a long period without further treatment, and then kill them and make a search for tuberculous lesions in their organs.

EXPERIMENTS WITH B. ANTHRACIS.

In this section will be described complement-fixation, precipitin, agglutination and bactericidal experiments with the sera of—two rabbits, Nos. 12 and 13, immunised with defatted anthrax antigen; a horse anti-anthrax serum (Mulford); and normal and various immune sera as controls.

I.—Complement-fixation Experiments.

By immunisation with "defatted" anthrax-antigen, complement-fixing substances are produced in definite though not great quantities Table (see XVII).

TABLE XVII.
COMPLEMENT-FIXATION TESTS. ANTHRAX.

DESCRIPTION OF SERUM	QUANTITY OF SERUM IN DROPS *	RELATIVE STRENGTH OF SERUM
NORMAL RABBIT 12, INJECTED WITH DEFATTED ANTHRAX ANTIGEN	2	2·5
NORMAL RABBIT 13 " " " " "	2	2·5
NORMAL RABBIT A	5	1·0
NORMAL RABBIT B	5	1·0
HORSE ANTI-ANTHRAX SERUM (MULFORD)	5	1·0
ANTIDIPHTHERIC HORSE-SERUM 400 UNITS	5	1·0
ANTIDIPHTHERIC HORSE-SERUM 500 UNITS	5	1·0
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	5	1·0

* I.E. THE SMALLEST QUANTITY OF SERUM WHICH, IN THE TECHNIQUE DESCRIBED ABOVE, GAVE COMPLETE COMPLEMENT-FIXATION.

It is of interest to note that the horse anti-anthrax serum, which has high agglutinating and precipitating properties, has no greater power of fixing complement than normal serum. It is also evident that anti-tuberculous and anti-diphtheritic horse sera do not fix the complement, and that, therefore, the effects obtained with the immunised rabbits are specific.

II.—Precipitin Tests. These are shown in Tables XVIII and XIX.

From Table XVIII it is seen that the anthrax precipitinogen, obtained by digesting defatted *B. anthracis* with trypsin, reacts specifically, since it gives a strong precipitation with the horse anti-anthrax serum (Mulford), but no reaction with normal rabbit serum nor with the serum of the tubercle-immune horse.

TABLE XVIII.
PRECIPITATION-EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM	HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION.	RELATIVE ACTIVITY OF SERA
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/256	256
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/1	1
NORMAL RABBIT 1	1/1	1
NORMAL RABBIT 2	1/1	1

The specificity of the precipitinogen having been demonstrated by means of the serum of an animal immunised with whole *B. anthracis*, it was important to discover whether the injection of "defatted" anthrax antigen into normal rabbits would produce specific precipitins.

TABLE XIX.
PRECIPITATION-EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM.	HIGHEST DILUTIONS OF PRECIPITINOGEN SHOWING EQUAL DEGREES OF PRECIPITATION.	RELATIVE ACTIVITY OF SERA
NORMAL RABBIT 12 } INJECTED WITH DEFATTED ANTHRAX ANTIGEN	1/32	32
NORMAL RABBIT 13 }	1/64	64
NORMAL RABBIT A.	1/1	1
NORMAL RABBIT B.	1/1	1
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/16	16
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/1	1

The experiments shown in Table XIX clearly prove this point, and show, in addition, that the sera of the rabbits immunised with "defatted" antigen are more active than the immune serum of the horse.

III.—Agglutination Experiments.

In Table XX the results of agglutination experiments, performed with a saline suspension of the "defatted" anthrax bacilli, are given.

The suspension was made up to a suitable opacity with normal saline formolised to 0·1 per cent.

TABLE XX.
AGGLUTINATION EXPERIMENTS. ANTHRAX.

DESCRIPTION OF SERUM	HIGHEST DILUTIONS OF SERA SHOWING EQUAL DEGREES OF AGGLUTINATION.	RELATIVE ACTIVITY OF SERA
NORMAL RABBIT 12 } INJECTED WITH DEFATTED ANTHRAX ANTIGEN	1/2·5	1
NORMAL RABBIT 13 }	1/50	20
NORMAL RABBIT 1	1/2·5	1
NORMAL RABBIT 2	1/2·5	1
HORSE ANTI-ANTHRAX SERUM (MULFORD)	1/250	100
HORSE IMMUNIZED AGAINST B. TUBERCULOSIS	1/2·5	1

From this table it is seen that the "defatted" antigen is agglutinated specifically by the immune anthrax-serum of the horse (obtained by immunising with the whole bacillus). It is also clear that the defatted antigen, on injection into normal rabbits, is capable of producing specific agglutinins. In the one rabbit, No. 12, no agglutinins had appeared at the time of testing, in spite of 3 or 4 injections with antigen, but the other (No. 13) responded well.

The point raised earlier in the paper, that there is no fixed relationship between the amounts of complement-fixing substances, precipitins and agglutinins contained in an immune serum, is brought out extremely clearly by a comparison of the results given in Tables XVII to XX. Thus it is found that the anti-anthrax horse-serum is the strongest in agglutination, takes a middle place as regards precipitation, and shows no complement-fixation in the quantity of serum used. While rabbit 12 is active in complement-fixation, and shows higher precipitating power than the horse immune serum, it betrays no agglutinating power in the quantities used.

Rabbit 13 gives definite complement-fixation and medium agglutination, while its precipitating power is of a high order.

IV.—Bactericidal Experiments *in Vitro.*

These experiments were carried out with two purposes:

(*a*) Of testing the theory that *B. anthracis* grown at 42° to 43° C. would be more sensitive than an ordinary culture grown at 37° C. to the bacteriolytic or bactericidal effects of normal human serum, and of the serum of a rabbit which had been injected with "defatted" anthrax-antigen.

(*b*) Of ascertaining whether the fresh serum of an animal immunised with defatted antigen contained bactericidal substances for a normal anthrax culture in greater quantities than those found in normal animals.

The details of the experiments are as follows:

Anthrax Bactericidal Experiment I.

Purpose.—To test the relative bactericidal effects of a normal human serum (A.D.G.) on anthrax bacilli grown at 37° C. and on the same culture grown at 42° to 43° C.

The *technique* was in the main that of A. E. Wright, but the results were measured by plating out a measured quantity of each test-mixture on agar-plates, instead of by observing at what dilution growth failed to occur.

The test-mixtures consisted of two volumes of the serum with one volume of a series of dilutions, in broth, of a well-shaken 24 hours' culture (at 37° C.) of an anthrax bacillus (No. I). The dilutions of culture ranged from 1 in 10 to 1 in 1,000,000, with a tenfold increase of dilution at each step.

A control for each mixture was made by substituting broth for the serum.

The mixtures were sucked into capillary pipettes, which, when the tips had been sealed, were incubated for 6 hours at 37° C. At the end of this time 10 cubic millimetres of each mixture were spread over the surface of agar plates, which were incubated for 24 hours.

Result 1/10 to 1/1000, all growths were confluent, but the general appearances suggested a denser growth of the 37° C. bacillus.

At a dilution of 1 in 10,000 the following results were obtained:

37° C. anthrax + serum . . .	About 80 to 100 colonies.
37° C. anthrax + broth . . .	About 500 colonies.
43° C. anthrax + serum . . .	1 colony.
43° C. anthrax + broth . . .	About 100 colonies.

Thus, the reduction in number of colonies under the influence of the serum was 15 to 20 times greater in the 43° growth than in the normal 37° culture.

It is to be noted that the 43° strain, which had been grown at about this temperature for 10 days with daily subcultures in broth, showed a definite decrease of Gram-positiveness.

Anthrax Bactericidal Experiment II.

Purpose.—To test the relative bactericidal effects of the serum of a rabbit immunised with "defatted" anthrax antigen on anthrax bacilli grown (1) at 37° C. and (2) at 42°–43° C.

Technique.—The culture-dilutions were made in sterile dwarf test-tubes with previously calibrated (0·1 c.c.) sterilised capillary pipettes in the following manner: Into a series of dwarf test-tubes 0·9 c.c. of broth was measured and the tubes were autoclaved just before use; 0·1 c.c. of the carefully shaken 18 hours' broth culture was added to the first tube and well mixed with the broth by sucking in and out; then 0·1 c.c. of the mixture was carried over into the second tube, and so on.

Dilutions of 1 in 10 to 1 in 1,000,000 were thus made with each culture (37° and 43° C., grown for 14 hours).

In a fresh series of dry sterile tubes mixtures were made of one volume of each culture dilution with one volume of the serum of a rabbit that had been immunised with "defatted" anthrax antigen. A separate series for the two cultures (37° and 43 C.) was thus set up, and for every tube of serum plus culture a control of broth plus culture, in identical dilution, was prepared.

The control tubes were plated forthwith, without incubation, while the mixtures of serum plus culture were incubated for 6 hours at 37° C. before being plated. The plates were made by spreading 0·02 c.c. of the mixture measured with a calibrated sterilised capillary-pipette on the surface of an agar plate.

The results are shown in Table XXI.

As far as the numbers of colonies are concerned, there seems to be little difference in the susceptibility of the two cultures, though what difference there is indicates greater destruction of the 42° C. bacilli. But a careful microscopical examination, including a count of the individual bacilli, showed that the 42° C. culture was composed of long chains averaging 15 bacilli per chain, while the 37° culture consisted of very short chains of about 3 elements on an average. Since each chain would be expected to start a colony, the number of bacteria destroyed must be reckoned as the number of chains destroyed multiplied by the number of bacilli per chain.

The number of bacilli of the 42° C. culture bacteriolysed must therefore have been some five times greater than the number of 37° bacilli.

TABLE XXI
BACTERICIDAL EXPERIMENT II

ANTHRAX CULTURE (37° AND 42° C.)							
SERUM OF RABBIT IMMUNIZED WITH DEFATTED ANTHRAX ANTIGEN							
DILUTIONS OF CULTURE	1/1	1/10	1/100	1/1000	1/10000	1/100000	1/MILLION
SERUM AND CULTURES 37° C.	ABOUT 1000	∞	SEVERAL THOUSAND	0	0	0	0
CONTROL OF CULTURES 37° C.	∞	∞	ABOUT 1000	168	20	2	0
SERUM AND CULTURE 42° C.	∞	∞	0	0	0	0	0
CONTROL OF CULTURE 42° C.	SEVERAL THOUSAND	ABOUT 1000	103	17	0	0	0

N.B. 37° AND 42° C. CULTURES WERE MADE FROM THE SAME COLONY OF AN AGAR PLATE · GROWN AT 37° C. CULTURE 37 – GRAM-POSITIVE; CULTURE 42 – GRAM-NEGATIVE – DENSITIES OF CULTURES SEEMED ABOUT EQUAL. THE FIGURES REPRESENT THE NUMBER OF COLONIES WHICH GREW.

Thus the conclusion is justified that growth at 42° C. lowered the resistance of the bacilli to the bactericidal action of this serum.

Anthrax Bactericidal Experiment III.

Purpose.—To compare the bactericidal action, on a normal anthrax culture grown at 37° C., of the serum of a rabbit immunised with defatted anthrax-antigen with that of a normal rabbit serum.

Technique.—*Mutatis mutandis*, the technique was precisely as in the last experiment.

Result.—With culture undiluted, normal serum gave a confluent growth while the immunised serum gave a semi-confluent growth. With culture 1/10, normal serum, about 1000 colonies; immunised serum, about 120 colonies. With culture 1/100, normal serum about 800; immunised serum, 0. With culture 1/1000, normal serum, about 70 colonies; immunised serum, 0.

With higher dilutions of culture no growth occurred in either series.

The serum of the rabbit immunised with "defatted" antigen thus showed itself to be enormously more powerful than the normal serum.

Summary of Conclusions from the Anthrax Bactericidal Experiments.

It has thus been shown in the case of *B. anthracis* that a Gram-positive bacterium, when grown at 42° to 43° C., tends to lose its power of retaining the Gram-stain, and that this change is accompanied by a lowered resistance to the bactericidal action of normal and immune sera.

These experiments afford substantial evidence in support of the explanation offered in the earlier part of this paper (p. 148) of Pasteur's fundamental experiments with "attenuated" anthrax virus.

EXPERIMENTS WITH OTHER BACTERIA.

With respect to bacteria other than *B. tuberculosis* and *B. anthracis*, experiments of a nature similar to those already described are in progress. A short description of the main results obtained up to the present will now be given.

The organisms have mainly been members of the Gram-positive group, *i.e. Staphylococcus aureus*, streptococcus and *B. diphtheriæ*, but a Gram-negative bacillus, *B. typhosus*, has also been subjected to experimental examination.

Staphylococcus aureus.

Defatted antigen has been prepared with this coccus in the manner already indicated. After formol-acetone extraction the coccus is Gram-negative.

It is then, but not before, digestible with trypsin, giving rise to a specific precipitinogen.

Injection of the "defatted" antigen into animals causes the production of complement-fixing substances, precipitins and agglutinins. Growth at 43° to 45° C. can produce a partial or complete loss of Gram-positive staining properties.

Streptococcus.

"Defatted" antigen has similarly been prepared. The "defatted" bacilli are Gram-negative and digestible with trypsin. There are at present no immunity experiments to record.

B. diphtheriæ.

Gram-negative, digestible, "defatted" antigen has been prepared. The digest gives a completely specific precipitin-reaction with antitoxic serum.

Antitoxic sera will also usually agglutinate saline suspensions of the "defatted" antigen. The antigen is specific when used for complement-fixation.

B. typhosus.

In order to show that the "defatting" process does not destroy the specific antigenic substances of a Gram-negative bacterium, *B. typhosus* was subjected to formol-acetone extraction, and rabbits were immunised with several doses of the resulting "defatted" antigen. All the sera thus produced gave positive complement-fixation reactions with the "defatted" antigen. Agglutinins, however, were formed only feebly, or not at all. With the specific precipitinogen, obtained by digesting the "defatted" antigen, the majority of the sera gave a much stronger precipitin-reaction than was shown by a powerful stock typhoid-agglutinating serum. The precipitinogen did not react with normal or immune sera of other types (*e. g. B. paratyphosus A*).

It may be noted that here, again, the absolute independence of agglutination and precipitation is very well demonstrated, which entirely corresponds with S. R. Douglas's findings with digests of acetone-extracted Gram-negative organisms.

TREATMENT OF HUMAN DISEASE.

The fact, now established, that "defatted" antigens of acid-fast, Gram-positive and Gram-negative bacteria could be digested with trypsin gave

strong support to the view that they would be similarly susceptible to the disintegrating action of the body-fluids, to which the bacteria, in their normal state, are, in varying degrees, refractory. Additional support to this expectation was given by the fact that the "defatted" antigens caused no local reaction when injected into normal animals.

It seemed, therefore, legitimate to hope that they would prove of value as antigens for the treatment of diseases such as tuberculosis, pyogenic infections, and refractory disorders caused by Gram-negative bacteria such as the gonococcus.

Having shown that small quantities of the antigens were devoid of toxic action, I felt justified in beginning to test their effect, in minute doses, on human patients, and in attempting to discover whether their action would be more favourable than that of the various tuberculins and of vaccines of the usual types.

The proof of such an action could clearly be obtained only by actual treatment of human beings, for animal experiments, however full and convincing, could only prove the case conclusively for the animal species concerned.

Early in April, therefore, acting on my suggestion, Dr. A. C. Inman, of the Brompton Hospital for Diseases of the Chest, inoculated with "defatted" T.B. antigen two patients suffering from active febrile tuberculosis of the lungs and pleuræ. The very small doses employed were determined according to the results of the animal experiments.

The patients, under the care of Dr. Bosanquet, had been under observation for a long period, and were both steadily getting worse. They were selected as being cases only likely to improve if some extra aid could be brought into application. Even in such active cases no local or focal reaction followed the initial inoculation, nor was there any appreciable febrile reaction. Encouraged by the result of the first few inoculations, it was decided to undertake the treatment of a case of acute toxic pulmonary tuberculosis in a young subject, aged 21 years. Again no local, focal or general reaction followed the small initial doses of the antigen; indeed, these resulted in lowering of the temperature and some amelioration of the general condition.

From the nature of the disease under consideration, patient judgment and the lapse of time will be necessary before any opinion can be expressed on the permanent value of the method of treatment. In due time, whatever be the result, a detailed account of all cases treated will be published.

Very shortly after Dr. Inman started treatment of his cases, Drs. P. Fildes and G. T. Western, at the London Hospital, who had followed the results of my researches for some time, and also Capt. S. R. Douglas of the Medical Research Council's Institute at Hampstead, kindly undertook in April of this year to investigate the therapeutic value of the "defatted" antigen of *B. tuberculosis* and certain other bacteria.

Thinking it desirable to avoid any expression of my personal opinion on the matter at the present moment, I have asked Drs. Fildes and Western to send me a short statement of their opinion as to the probable value of this form of treatment, so far as they are able to judge on their present experience. They have been given facilities for testing these antigens at the London

Hospital by the House Committee of that Hospital. Their report is here given verbatim:

"We have under treatment with Dreyer's antigen 60 cases of tuberculosis. Among these are 36 cases of tuberculous adenitis, 11 cases of cutaneous tuberculosis, 4 cases of pulmonary tuberculosis, both active pyrexial and apyrexial, 3 cases of joint and bone tuberculosis, 1 case of peritonitis, 1 case of tuberculosis of the urinary tract, 1 case of iridocyclitis and 3 cases of epididymitis. Most of these cases have been under our personal observation for considerable periods, up to 5 years, and have been selected for treatment as having shown little improvement under treatment with tuberculin B.E. (Koch).

"Improvement has taken place in nearly all cases, and is, in our opinion, of an order which exceeds obviously that obtainable by any other form of treatment which is applicable to these conditions.

"We have not observed any toxic effect following on the inoculations.

"With regard to the effect of these antigens on other bacterial infections, we have treated 6 cases of streptococcal infection such as septicæmia, erysipelas and osteomyelitis, 17 cases of staphylococcal infections, such as sycosis barbæ, furunculosis, etc., and 5 cases of gonorrhœal infection.

"Undoubtedly the course of the disease in many of these cases has been satisfactory as compared with previous experience with vaccine-treated cases. The issue, however, has not always been so straightforward, and therefore an opinion based on three weeks' experience must be more reserved.

"In due course a detailed account of these cases will be published."

In conclusion, I wish to express my warmest thanks to Mr. R. L. Vollum, who has given me assistance of the utmost value and in the most disinterested manner with the preparation of antigens and the performance of the tests.

Also to Dr. A. D. Gardner, who has helped me in a number of ways with experiments, and in the accelerated production of this paper. Further, to Dr. H. K. Ward, who also gave kind help in many experiments; and to Mr. Jesse Wheal, for his careful, skilful and painstaking care of the animals over many months.

In addition, I wish to express my gratitude to Capt. S. R. Douglas, F.R.S., for his helpful interest and invaluable assistance both in animals and materials.

Finally, though not least, to Dr. A. C. Inman, Dr. P. Fildes and Dr. G. T. Western, for their keen interest and their willingness to apply their skill and experience to the testing of my antigens on cases of human disease.

I have also to acknowledge the liberal assistance given to me by the Medical Research Council. Their financial help has greatly facilitated my work.

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